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THE EFFECTS OF ACTH ON THE METABOLISM OF RADIOACTIVE CALCIUM IN NORMAL AND HYPOPHYSECTOMIZED RATS*

FRANK ULRICH, D. HAROLD COPP, C. WILLET ASLING, CHOH HAO LI AND WILLIAM O. REINHARDT

From Department of Biochemistry, Divisions of Physiology and Anatomy, University of California, Berkeley and Division of Physiology, University of British Columbia, Vancouver

INTRODUCTION

IT was shown by Becks, Simpson, Li, and Evans (1944a and 1944b) and later confirmed by Baker and Ingle (1948) that ACTH causes a retardation of chondrogenesis and osteogenesis in the tibias of immature and adult rats. The following experiment was carried out to study the metabolism of radioactive calcium (Ca^{45}) in intact and hypophysectomized rats which were in growth stasis as a result of treatment with ACTH.

MATERIALS AND METHODS

Animals used in this experiment were male rats of the Long-Evans strain. The procedures and preparation of the animals are described in the previous paper by Asling, Reinhardt, and Li (1950). The following groups were compared: (1) intact controls, (2) intact, ACTH-treated, (3) hypophysectomized controls, and (4) hypophysectomized, ACTH-treated.

After injection for 39 days (from the 22nd to the 60th day of age), each rat was injected intraperitoneally with approximately 10 micrograms of calcium tagged with 6 microcuries of Ca^{45}. The calcium was in the form of $CaCl_2$ dissolved in 0.25 cc. of a 0.9 per cent solution of saline.

After injection each rat was placed in a large glass beaker which was provided with a wire screen for separation of the urine and feces. One hour later the rat was sacrificed with chloroform. Serum was obtained from blood drawn by heart puncture. A suitable aliquot of serum was placed on an aluminum disc and the radioactive calcium present determined with a thin window Geiger-Mueller counter. Due to the very soft beta radia-

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tion which Ca^{45} emits, suitable corrections were made for self-absorption. The total amount of radioactive calcium in the serum of the intact animals was calculated from the value of 2.43 cc. of serum per 100 grams of body weight reported by Berlin, *et al.*, (1949). The value of 2.76 cc. of serum per 100 grams of body weight, observed by Berlin and co-workers (1950), 37 days after hypophysectomy, was used to calculate the total serum Ca^{45} in the operated animals.

The inside of the beakers and the wire screens were thoroughly rinsed with 1N HCl and the resulting washings added to the previously collected urine. The bladder was washed out with 1N HCl and these washings were also added to the urine samples. A suitable aliquot was then analyzed for Ca^{45}.

The dry and ash weight of the femurs was determined. The bones were then dissolved in hot 1N HCl, made up to volume with distilled water, and the amount of radioactive calcium present determined in a manner similar to that of the serum and urine.

Radioautographs were prepared to demonstrate the distribution of radioactive calcium in the tibia of each animal. The tibias were frozen at −20 degrees Centigrade and the proximal portion of the bone was split longitudionally with a razor blade exposing the marrow cavity and the epiphyseal cartilage plate. The split, frozen tibias were then affixed with rubber cement to a piece of fine-grained sponge rubber which fitted into a small wooden slide box. Kodak industrial type A safety X-ray film which had been chilled beforehand was placed in contact with the bones. The cover was placed on the slide box with a lead brick on top in order to maintain close contact between the film and the bones. The specimens were kept frozen at −20 degrees Centigrade for 102 hours after which the film was removed and developed. Because of the soft beta radiation of Ca^{45} rather good definition was obtained despite the thickness of the sections.

RESULTS

The data are summarized in Table 1. One hour after intraperitoneal injection the serum contained between two and three per cent of the

TABLE 1. THE Ca^{45} CONTENT OF THE SERUM, FEMUR, AND URINE ONE HOUR AFTER THE INTRAPERITONEAL INJECTION OF RADIOACTIVE CALCIUM INTO 60-DAY OLD MALE RATS, INTACT AND HYPOPHYSECTOMIZED, INJECTED WITH ACTH FOR 39 DAYS: INCLUDING ALSO THE DRY WEIGHT, ASH WEIGHT, AND ASH WEIGHT/DRY WEIGHT RATIO OF THE FEMURS

		Serum	Femur				Urine
Experiment	No. of animals	Per cent dose	Per cent dose uptake	Dry weight grams	Ash weight grams	Ash Weight / Dry Weight	Per cent dose
Intact Controls	5	2.19±0.08*	2.37±0.12	0.415±0.019	0.206±0.009	0.50±0.01	0.06±0.01
Intact, ACTH-Treated	4	2.30±0.29	2.20±0.28	0.293±0.020	0.148±0.012	0.51±0.01	0.85±0.27
Hypophysectomized Controls	4	2.88±0.32	1.82±0.16	0.141±0.005	0.071±0.003	0.50±0.01	1.22±0.42
Hypophysectomized, ACTH-Treated	4	2.34±1.03	0.72±0.17	0.125±0.006	0.061±0.004	0.48±0.01	0.72±0.26

* Mean ± standard error

injected dose in each group of animals. No significant difference between any two groups could be shown.

There was no significant difference in the total Ca^{45} content of the

femur between the intact controls and the intact treated animals. Although the radio-calcium uptake in the femurs of the hypophysectomized controls was lower than in the intact controls, the differences were not statistically significant. Treatment with ACTH did not appear to effect the uptake of Ca^{45} in the intact animals but did cause a very significant reduction of uptake in the hypophysectomized rats.

The ash weight/dry weight ratio of the femurs from each group were consistently the same and ranged from 0.48 to 0.51.

Despite the fact that the average Ca^{45} urinary excretion in the intact,

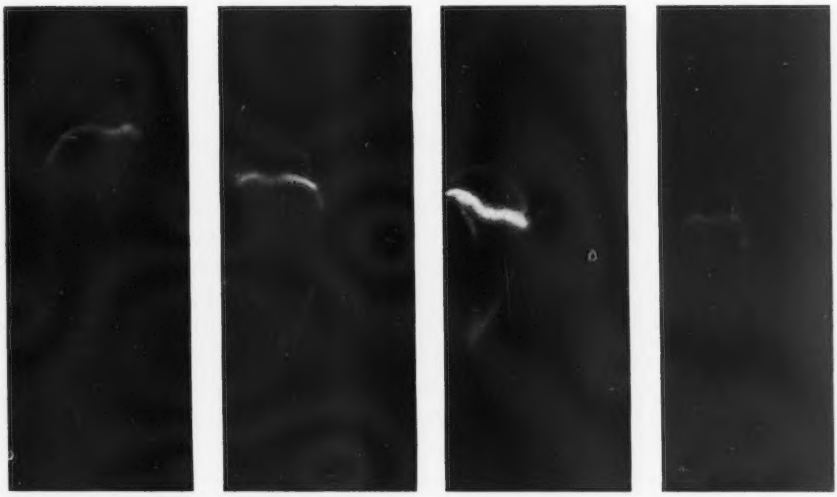

FIG. 1. Radioautographs of split rat tibias one hour after the intraperitoneal injection of a tracer dose of Ca^{45}; in order from left to right: intact control, intact, ACTH-treated, hypophysectomized control, and hypophysectomized, ACTH-treated.

ACTH-treated animals was fourteen times greater than in the intact controls, there was such individual variability in both groups that it was not possible to demonstrate any statistical significance in this. This variability was probably due to the short time interval during which the urine was collected since it is very difficult to obtain comparable samples during a one hour collection period. There was no apparent difference in the urinary excretion of radioactive calcium between the hypophysectomized controls and the hypophysectomized, ACTH-treated animals. Although the urinary Ca^{45} excretion in the hypophysectomized controls was twenty times greater than in the normal controls, again the variability among individuals precluded demonstration of a statistically significant difference.

Representative radioautographs of the tibias from the four groups are shown in Figure 1. They reflect to some extent the differences in the Ca^{45} content of the femurs. There was a less intense image of Ca^{45} in the tibias of the treated hypophysectomized rats than in the hypophysectomized controls. The latter also showed an intense line of deposited Ca^{45} in the epiphyseal plate region. This would correspond to the site of the sealing off bone which has been shown to develop at about this time in hypophysectomized rats (Becks, Simpson, and Evans 1945), and which has not been seen following ACTH treatment (Becks, Simpson, Li, and Evans 1944a and 1944b). The apparent lesser intensity in the tibias of the intact animals may be explained by the larger size of the bones and the greater areas over which the Ca^{45} was distributed.

SUMMARY

1. The effects of ACTH on the calcium metabolism of intact and hypophysectomized male rats was studied with Ca^{45}. The animals were sacrificed one hour after intraperitoneal injection of the radio-calcium, having received ACTH injections from the 22nd to the 60th day of age.

2. No significant differences were found in the serum levels of Ca^{45}.

3. ACTH treatment did not appear to effect the uptake of radio-calcium by the femurs of the intact animals but did significantly reduce the uptake in the hypophysectomized group.

4. The urinary excretion of the intact controls was lower than in any of the experimental groups. However the variability in the data was such that these differences were not statistically significant.

5. Radioautographs were obtained showing the Ca^{45} distribution in split tibias. In the hypophysectomized controls a heavy line of deposited Ca^{45} was observed in the epiphyseal plate region, contrasting with the lesser uptake observed in this region in rats of the other three groups.

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DISAPPEARANCE RATE OF EXOGENOUS THYROTROPHIN FROM THE BLOOD OF NORMAL AND HYPOPHYSECTOMIZED RATS[1,2]

SAVINO A. D'ANGELO

From the Department of Anatomy, Jefferson Medical College, Philadelphia, Pa.

THE metabolic fate of the thyrotrophic hormone remains conjectural despite the many experiments establishing the fundamental nature of the interplay between thyroid and anterior hypophysis. The general uncertainty regarding the detection and the quantitative estimation of thyrotrophin in blood and urine (see Collip, 1942; Adams, 1946; Albert, 1949; D'Angelo and Gordon, 1950; for extended consideration of this problem) and the frequent failure to recover thyrotrophin in the body fluids even after high dosage administration appear to have presented major difficulties in metabolic studies of the thyrotrophic hormone. Certain studies have yielded provocative results, however. Loeser (1934) reported the detection of intravenously administered thyrotrophin in the blood and urine of normal and thyroidectomized rabbits, with injected hormone disappearing sooner from the blood in normal than in thyroidectomized animals. Results of in-vitro and in-vivo experiments by Seidlin (1940) on the metabolism of thyrotrophic hormone in the guinea pig were considered to suggest that the thyroid gland plays a major role in the withdrawal of thyrotrophin from the circulation.

Recent experiments with the stasis-tadpole technique (D'Angelo and Gordon, 1950) have demonstrated that minute quantities of thyroid and thyrotrophic hormones added to raw serum can be bioassayed simultaneously in the same sample, and, further, that the method is sufficiently sensitive to detect the small concentrations of thyrotrophin which normally exist in the body fluids. Accordingly, it has been found possible to determine the thyroid-thyrotrophin balance in the circulating blood of various species under normal, experimental, and clinical conditions (D'Angelo *et al.* 1949a, 1949b, 1950). The present investigation utilizes the method to ascertain, as a prerequisite step for the study of thyrotrophin metabolism, the rate of disappearance of a single dose of administered hormone from the circulating blood of normal and hypophysectomized rats.

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[2] I wish to acknowledge gratefully technical assistance from Mrs. Ada Schafer of the technical staff, at Jefferson Medical College.

MATERIALS AND METHODS

Hypophysectomized male rats (150–170 gms.; 3–7 weeks postoperative and with all "target" organs atrophic) of the Sprague-Dawley strain, and intact control rats of the same age (250–325 gms. body weight) were used. The purified thyrotrophin[3] was administered to the animals by intracardial injection after light ether anesthesia. Rats of both the intact and hypophysectomized groups received 1 mg. of the hormone dissolved in 1 cc. of saline, excepting those intact animals whose body weights exceeded 300 gms. In these the quantity of hormone was adjusted on a weight basis, to be equivalent to that administered to the hypophysectomized partners. After various time intervals (5–7 mins.; 1 hr., 5 hrs., 20–24 hrs. and 46–48 hrs.), the experimental animals were reanesthetized with ether and blood was withdrawn in approximately equal amounts (4–6 cc.) from rats in each respective group and pooled. Blood was withdrawn without anti-

TABLE 1. BIOASSAY OF PURIFIED THYROTROPHIN[1] IN THE STASIS TADPOLE

Dosage	No. of Injections	Metamorphic Changes: Mean hindlimb Increase (mm.)	Metamorphic Changes: Body	Mean Thyroid Cell Hgt. (μ)
200 μg.[2]	4	6.0 ± 0.5[3]	Tail Resorption[4]	10.2 ± 0.3[5]
20.0 μg.	4	5.6 ± 0.4	Fore limb[4] Extrusion	10.1 ± 0.2
2.5 μg.	5	4.6 ± 0.2		7.8 ± 0.2
0.4 μg.	5	3.6 ± 0.2		7.2 ± 0.1
0.25 μg.	5	2.1 ± 0.2		6.8 ± 0.2
0.085 μg.	6	1.0 ± 0.5		5.9 ± 0.1
Control (saline)	5	0.2 ± 0.1		5.2 ± 0.1

[1] Parke, Davis preparation Rx-099931.
[2] Total amount of hormone given over entire injection period.
[3] Hindlimb increase (±standard error of mean) over initial mean hindlimb length (similar in all groups; 3.3–3.8 mm.).
[4] The superimposition of these developmental events on hindlimb extension indicates maximal acceleration of the metamorphic process.
[5] Average mean thyroid cell height (micra ± standard error of mean) based on measurements in, at least, 6 animals of each group.

coagulant by direct cardiac puncture. In animals at the 5–7 minute time interval, however, withdrawal was made from the inferior vena cava. Sera obtained from the pooled bloods were kept refrigerated and allowed to thaw only during the injection period incident to thyrotrophic hormone assay.

The bioassay of thyrotrophin was done with the stasis-tadpole technique (full details have already been given; D'Angelo *et al.*, 1942, 1950). A standard dosage curve for the purified thyrotrophin preparation used was obtained by serial dilution of the hormone in saline and injection into groups of stasis tadpoles. Test animals were given a total dose of hormone ranging from 0.085 to 200 μg. over the entire injection period (4–6 injections; 0.05 cc. given on alternate days). Thyroid cell height was measured micro-histometrically and the metamorphic advance was assessed by hindlimb increase and other developmental criteria (emergence of fore limbs; tail resorption). After the standard nomogram had thus been described, the various sera from the hormone-treated rats were similarly

[3] Parke, Davis, preparation RX-099931; generously supplied through the courtesy of Mr. L. W. Donaldson.

tested in stasis tadpoles to ascertain the degree of recovery of the administered thyrotrophin. Each test animal received the same volume of serum (0.2 cc.) and the same number of injections (4 injections of 0.05 cc. on alternate days).

RESULTS

The data obtained from the bioassay experiments establishing the standard response with pure thyrotrophin are summarized and graphically

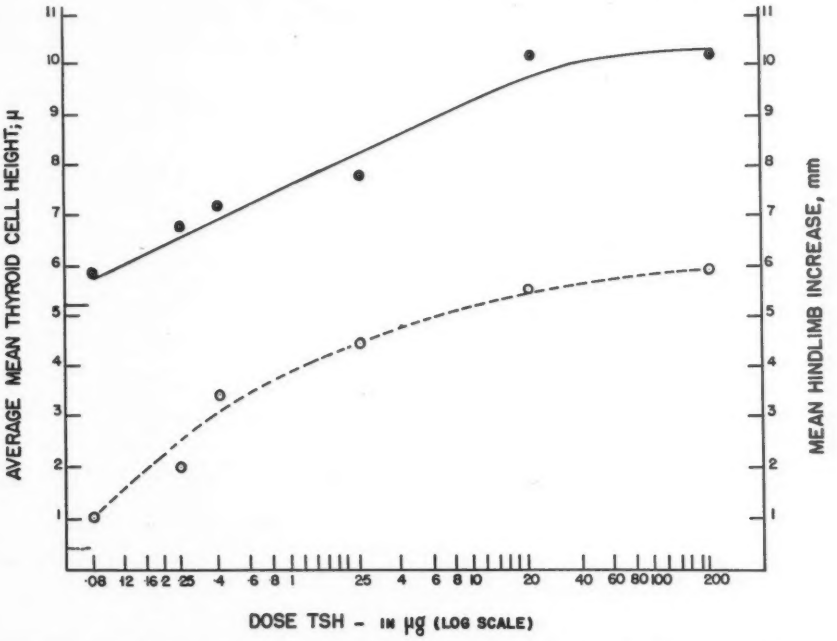
AVERAGE MEAN THYROID CELL HEIGHT; μ
MEAN HINDLIMB INCREASE, mm
DOSE TSH - IN μg (LOG SCALE)
·08 12 16 2 25 ·4 ·6 8 1 25 4 6 8 10 20 40 60 80 100 200

FIG. 1. Log dose-response curve of purified thyrotrophin (TSH) Parke, Davis; Rx-099931 showing the increase in thyroid cell height (solid line), and hindlimb increase (broken line) in the stasis tadpole. Cell height at each point based on counts in 6–8 animals.

Horizontal bars on ordinate represent values in control animals.

shown in table 1 and figure 1. It is seen that thyroid cell height was significantly elevated over control values with 0.085 μg. of the preparation. Cell height increased progressively with higher doses to achieve peak values at the 20 and 200 μg. levels. The thyroid stimulation was accompanied by progressive hindlimb length increase, and with higher doses, by other manifestations of accelerated development (fore limb emergence, tail resorption). The control stasis tadpoles gave no indication of any resumption of their arrested development, and the thyroids of these ani-

mals were typically resting with distended follicles and flattened epithelium. These results are essentially similar to those previously described for the bioassay of International Standard Preparation of Ox Pituitary (D'Angelo and Gordon, 1950).

The results of the thyrotrophin assays of rat sera obtained from the bloods of animals taken at various time intervals after a single intracardial injection of the hormone are presented in table 2 and figure 2. It is evident that the sera of untreated, or saline treated intact rats, possessed thyroid stimulating activity as indicated by thyroid cell height elevation in the

TABLE 2. DIFFERENTIAL RECOVERY OF INTRACARDIALLY ADMINISTERED THYROTROPHIN IN SERUM OF NORMAL AND HYPOPHYSECTOMIZED RATS

Group	Mean body Wgt.—gm. (# of Ans.)	Bleeding time after injection	Effect of sera on stasis tadpole[1]			Conc. thyrotrophin[4] detected; μg./ml. serum	Mean[5] recovery of thyrotrophin (%)
			Hindlimb[2] increase	Body	Thyroid[3] cell hgt.		
Normal	321 (3)		2.9±0.2		6.3±0.1	0.6	
Normal, Sal.	263 (3)	5–7 mins.	2.8±0.3		6.3±0.2	0.6 (0.5–0.9)	
Hypx., Sal.	158 (3)	5–7 mins.	0.1±0.1		5.1±0.1	0	
Normal, Thyrotrophin[6]	247 (3)	5–7 mins.	7.1±0.6	50%[7]	9.2±0.3	50 (30–70)	29.2
Normal, Thyrotrophin	260 (2)	1 hr.	5.3±0.4	30%	7.6±0.2	5 (4–6)	2.7
Hypx., Thyrotrophin	153 (3)	1 hr.	6.6±0.4	30%	9.5±0.2	70 (50–110)	25.9
Normal, Thyrotrophin	260 (2)	5 hrs.	5.2±0.4		7.0±0.3	2 (1–3)	0.9
Hypx., Thyrotrophin	157 (3)	5 hrs.	7.8±0.5	50%	8.7±0.2	25 (20–30)	9.5
Normal, Thyrotrophin	265 (3)	20–22 hrs.	2.2±0.1		6.1±0.1	0.5 (0.4–0.6)	0.0
Hypx., Thyrotrophin	145 (2)	23–24 hrs.	2.1±0.2		6.1±0.2	0.5 (0.4–0.6)	0.2
Normal, Thyrotrophin	321 (3)	46–47 hrs.	2.3±0.1		6.1±0.1	0.5 (0.4–0.6)	0.0
Hypx., Thyrotrophin	169 (2)	46–47 hrs.	1.0±0.1		5.5±0.2	0	

[1] Each test animal in all groups received a total of 0.2 ml. serum over 4 injections.
[2] Mean hindlimb increase in mm. ± standard error of mean (initial hindlimb lengths similar in all test groups).
[3] Average mean thyroid cell hgt. in micra ± standard error of mean.
[4] Estimated from standard Nomogram; in parentheses, values as calculated from standard error of each assay.
[5] Represents: $\frac{\text{Conc. Thyrotrophin detected—Conc. of Endogenous Thyrotrophin}}{\text{"Expected" Thyrotrophin Conc.}} \times 100$
[6] Dosage—1 mg. in each animal of all hormone treated groups, except in the normal 46–47 hr. group which received 1.8 mg. of Thyrotrophin.
[7] The percentage of test animals showing extrusion of fore limbs.

test tadpoles from the control value of 5.2 ± 0.1 μ to 6.3 ± 0.2 μ. By the extrapolation of this cell height value on the standard curve (Fig. 1) it may be calculated that the mean concentration of endogenous thyrotrophin in normal rats of this study, approximated 0.6 micrograms thyrotrophin per milliliter of serum (i.e., 0.12 μg.$\times 1/0.2$) with a range of 0.5–0.9 μg. as estimated from the standard error of the mean. It is seen further that the sera of saline treated hypophysectomized animals, in contrast, did not display any measurable thyrotrophic hormone activity since thyroid cell height in test tadpoles receiving these sera did not exceed that in the controls. The lack of thyroid stimulation could be correlated with the absence of hindlimb growth or other signs of developmental advance in the test animals.

It is equally clear from these data that a single dose of intracardially administered thyrotrophin is recoverable in the blood of both intact and

hypophysectomized animals within certain time limits after injection. In intact rats, the mean concentration of thyrotrophin detected at the average 6 minute interval was calculated to be 50 μg. per milliliter of serum (range, 30–70 μg.), representing a recovery of approximately 29.2% of the "expected" concentration. (The expected concentration is calculated on the assumption that the 1,000 μg. of administered thyrotrophin in both intact

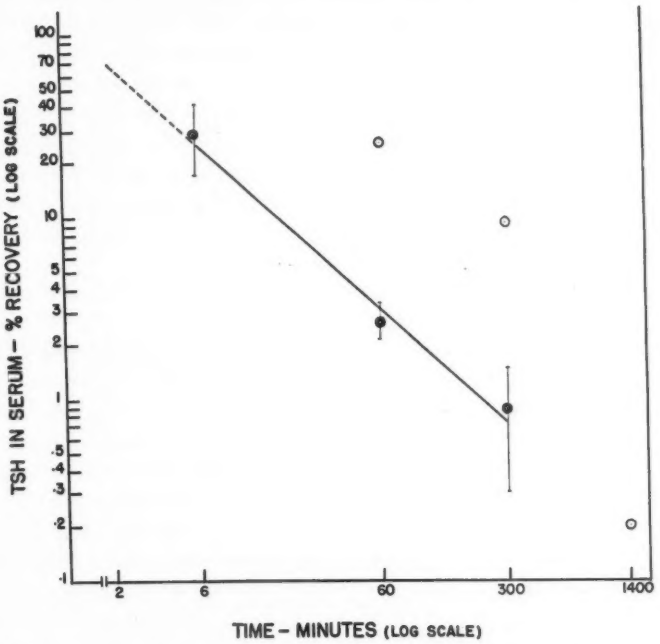

FIG. 2. Disappearance of a single dose of intracardially administered thyrotrophin (TSH) from rat sera. The curve depicts the rate of disappearance of the hormone in the intact animal. The open circles represent mean thyrotrophin recoveries at corresponding time intervals in the sera of hypophysectomized animals.

and hypophysectomized animal are distributed in a plasma volume equivalent to approximately 2.4% of the body weight; Berlin *et al.* Plasma volume difference between intact and hypophysectomized rats is not of sufficient magnitude to invalidate the comparison of thyrotrophin recoveries made in both groups.) At the end of one hour only 2.7% of the expected concentration of thyrotrophin could be detected in the serum of intact animals. Recovery values at the end of the 5th hour indicated that approximately 99% of the exogenous thyrotrophin had vanished from the circulation by this time. Examination of the graph (Fig. 2) reveals that

the rate of disappearance of the hormone in the intact rats is best described by a logarithmic type of curve. Extrapolation of the curve toward zero time yields theoretical recovery values (70% at the two minute interval) which suggest that practically all of the administered hormone might be recoverable at the zero time interval.

The results obtained with the sera of hypophysectomized rats clearly indicate that the injected hormone disappeared from the blood at a slower rate than in intact animals. At the end of the first and fifth hours, the mean thyrotrophin recoveries in serum were 25.9% and 9.5% respectively. Thus, the hormone concentrations in the blood of the hypophysectomized animals were approximately 10 fold greater than those in hormone injected intact animals at comparable time intervals. An appreciation of the slower rate of hormone disappearance in the hypophysectomized animal is gained from the fact that the 1 hour recovery value approximated the 5–7 minute interval value in the intact animal (this interval was not tested in the hypophysectomized animal).

DISCUSSION

Investigations designed to ascertain the rate of disappearance and the metabolic fate of pituitary trophic hormones in the organism have been so few that generalizations in this area of endocrine investigation are not too profitably made. It is interesting, nevertheless, to compare directly the present findings utilizing thyrotrophin with those of Greenspan, Li, and Evans (1950) in experiments with adrenocorticotrophin. In both studies, purified trophic hormones, previously standardized by sensitive methods of bioassay, were administered directly into the circulation of the same laboratory animal and their disappearance rates from the blood precisely determined. Although both of these studies are admittedly preliminary in nature, the rather ephemeral existence of adrenocorticotrophin and thyrotrophin in the circulation of the intact animal is clearly indicated. The results suggest, moreover, that of the two trophic hormones thyrotrophin is less fleeting in its existence than is adrenocorticotrophin. Approximately 98% of the injected dose of hormone had disappeared after 6 and 60 minutes for adrenocorticotrophin and thyrotrophin respectively. It appears significant that only 6% of the injected dose of adrenocorticotrophin was theoretically recoverable in the plasma at zero time whereas similarly extrapolated values indicate almost complete recovery of thyrotrophin. Interpretation of these differences between the thyrotrophic and adrenocorticotrophic hormone activity in blood is better undertaken when the activities of the respective target organ hormones in the circulation are similarly investigated.

It is probably not fortuitous that the removal of thyrotrophin from the

blood in the hypophysectomized animal, like that in the thyroidectomized animal (Loeser, 1934), proceeds at a slower rate than in the intact animal. In Loeser's experiments administered thyrotrophin could be detected in the blood of the thyroidectomized rabbit several hours after it had vanished from that of the intact animal. The withdrawal of thyrotrophin from the circulation could not be accounted for by storage in such tissues as liver, spleen, kidney, and muscle, and it was postulated that the persistence of the hormone in the blood of the thyroidectomized animal was due either to decreased urine elimination or to the absence of the thyroid gland which normally might inactivate or remove thyrotrophin from the circulation. Seidlin (1940) was inclined to believe that the thyroid gland played a considerable part in withdrawing thyrotrophin from the blood since the hormone was partly removed from solution in contact with a suspension of beef thyroid tissue, and because administered thyrotrophin was recoverable in the urine of thyroidectomized guinea pigs but not in similarly treated intact animals. It must be pointed out that in experiments with thyroidectomized animals a strong possibility exists that endogenous thyrotrophin from the now unrestrained adenohypophysis may increase in sufficient amounts in the blood to augment the levels of the exogenous thyrotrophin. The hypophysectomized animal thus is better indicated in studies such as these since, as has been shown, no endogenous thyrotrophic hormone is detectable in the blood.

Recovery of thyrotrophin in the urine has not been attempted in these experiments, nor has water balance been studied; consequently, it cannot be stated whether or not the slow rate of disappearance of the hormone from the blood of the hypophysectomized animal is attributable largely—or even in part—to delayed excretion rates. There is considerable evidence to indicate that marked disturbances in water metabolism do characterize the thyroidectomized or hypophysectomized animal (Gaunt, Birnie, and Eversole, 1949). It is considered more plausible, nevertheless, that the persistence of administered hormone in the circulation of the thyroidectomized or hypophysectomized animal rather reflects a retardation in the rate of destruction of the hormone due to the lowered oxidative metabolism of the tissues (including the thyroid). The concomitant administration of various calorigenic agents with thyrotrophin in the intact and endocrine-deficient animal should yield pertinent information in this regard.

SUMMARY

The level of thyrotrophin in the blood of intact and hypophysectomized rats was determined at various time intervals after a single intracardial injection of the hormone.

The rate of hormone disappearance was found to be appreciably greater

in normal than in hypophysectomized animals. In intact animals, the serum concentration of thyrotrophin tended to fall logarithmically against time, with less than 3% and 1% of the injected dose detectable after 1 and 5 hours respectively. Thyrotrophin levels in the blood of hypophysectomized rats were approximately 10 fold greater at similar time intervals, and the hormone was still present in the circulating blood 24 hours after injection.

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THE INFLUENCE OF THYROID HORMONE AND ANDROGEN ON COMB GROWTH IN THE WHITE LEGHORN COCKEREL*

DAVID M. MORRIS[1]

From the Zoology Department, Indiana University,[2] Bloomington, Indiana

INTRODUCTION

IT has been shown (Caridroit, 1941; Simmons, 1943; Blivaiss, 1947, and others) that comb growth is partly dependent upon the presence of thyroid hormone. Simmons reported that the growth of the comb in the White Leghorn capon could be increased by the administration of thyroxine and that the effect was roughly proportional to the dose. Blivaiss presented data showing a greater increase in growth of combs of Brown Leghorn capons and thyroidectomized males following the simultaneous administration of thyroxine and testosterone propionate than with either alone. Data submitted in the present paper substantiate some of the earlier work and support the suggestion of a synergistic action of thyroid hormone and androgen in the promotion of comb growth in the White Leghorn fowl.

MATERIALS AND METHODS

Changes in the weight of the comb, thyroids, and gonads, in response to various treatments designed to control the level of circulating thyroid hormone and androgen were studied. Animals were subjected to one of the following experimental conditions: (1) limited diet, (2) thyroidectomy, (3) thyroidectomy and castration. (4) thyroidectomy with injections of testosterone propionate, (5) administration of thiouracil and testosterone propionate. Limited diet was established as that amount of standard chicken mash equivalent to half that consumed by the control animals. The control birds had food before them at all times while the experimental birds were fed at once half the amount consumed by the control animals the previous day. Thiouracil powder[3] was mixed in the appropriate amounts with regular chicken mash to prepare the proper concentrations as indicated in a later section. A quantity sufficient to last for about 7 to 14 days was prepared at one time. Testosterone propionate in peanut oil[4] was prepared by

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[1] Present address: Biology department, North Texas State College, Denton, Texas.

[2] Contribution No. 410 from the Zoology department, Indiana University.

[3] Thiouracil powder was supplied through the courtesy of the Lederle laboratories.

[4] The author is indebted to the Schering corporation for their contribution of the testosterone propionate used in this study.

diluting with Sesame oil. All injections were made subcutaneously in the cervical region. Control birds for androgen-treated series received injections of Sesame oil.

RESULTS AND DISCUSSION

(1) Effect of limited diet

In the three age levels tested a slower rate of comb growth was observed in the experimental birds (Table 1). This was attributed, in part, to a lowered level of androgen induced by the restricted diet suggested by the lowered growth rate of the gonad in these series. Breneman (1940) and

TABLE 1. EFFECT OF LIMITED DIET ON WEIGHT OF THYROIDS, GONADS AND COMB

Treatment	Body		Thyroids		Gonads		Comb	
	No.	gms.	No.	mgs.	No.	mgs.	No.	mgs.
Treatment begun at 4 days of age								
LD 2 days	10	44	10	2.62	10	24.60	10	21
ND	10	48	10	3.75	10	25.70	10	22
LD 4 days	15	47	15	3.46	14	19.00	15	20
ND	10	55	10	4.10	10	20.20	10	17
LD 11 days	17	65	12	5.70	17	19.38†	17	28†
ND	17	82	12	6.20	17	35.24	17	54
LD 25 days	21	108	16	7.20†	21	18.70†	21	54†
ND	20	218	15	13.70	20	33.12	20	224
Treatment begun at 22 days of age								
LD 7 days	14	171	9	11.40	14	20.18*	14	102
ND	13	243	8	15.70	13	26.79	13	200
LD 14 days	14	192	9	10.80†	14	22.08*	14	161*
ND	14	320	8	20.60	14	28.78	14	356
LD 28 days	17	364	12	22.60†	17	30.76*	17	321†
ND	17	540	12	50.01	17	66.11	17	852
LD 42 days	13	545	7	31.75†	13	104.40‡	13	908
ND	12	815	7	57.80	12	203.90	12	1418
Treatment begun at 58 days of age								
LD 7 days	18	596	15	45.20	18	83.72	18	1120
ND	18	678	15	55.00	18	93.95	18	1156
LD 14 days	17	652	14	43.60†	17	28.07*	17	830
ND	17	840	14	74.10	17	160.95	17	1100
LD 28 days	15	786	12	57.50†	15	29.13†	15	967
ND	19	1082	16	84.10	19	165.16	19	1396
LD 42 days	18	871	13	64.20†	18	36.28†	18	1458
ND	18	1300	13	113.70	18	143.38	18	1838

LD Limited diet. ND Normal diet.
Weights of gonads and combs are expressed as mgs. per 100 gms. body weight.
*† Difference from the ND control significant at the 5% and 1% level respectively.
‡ In the ND series $s = 63.7$; in the LD series $s = 34.4$. This probably accounts for the high P value. Recalculation after omission of two widely varying figures in each series using Wright's rejection criterion, revealed a significant difference at the 5% level.

Payne (1946) reported a similar inhibition of gonad growth by restricted diet in White Leghorns and correlated it with a lowered gonadotrophic activity of the pituitary. However, since the thyroids in the limited-diet birds weighed less than those of the birds on regular diet in all the series tested, it was felt that a lowered thyroid hormone level may also have contributed to the observed retarded comb growth. Results of the following experiments support this hypothesis.

(2) Effect of thyroidectomy

In an attempt to evaluate the influence of the thyroid three groups of birds were studied as follows: (1) totally thyroidectomized (2) partially

TABLE 2. EFFECT OF TOTAL AND SUBTOTAL THYROIDECTOMY ON GONAD AND COMB WEIGHTS IN COCKERELS AND CAPONS. (40–43 DAYS OLD)

Treatment	No.	Body gms.	Gonads mgs.	Comb mgs.
Controls	36	374	33.19	507
Subtotal thyroidectomy	21	270	27.19	191*
Total thyroidectomy	5	128	19.41‡	15*§
Castration	11	433	—	128†
Castration, subtotal thyroidectomy	10	327	—	81†‖
Castration, total thyroidectomy	3	219	—	82

Gland and comb weights are expressed as mgs. per 100 gms. body weight.
*† Difference from the control significant at the 5% and 1% level respectively.
‡§ Difference from the subtotally thyroidectomized series significant at the 5% and 1% level respectively.
‖ Difference from the castration series significant at the 1% level.

thyroidectomized (3) unoperated controls. All experimental animals were operated on between the first and sixth day of age and were sacrificed on the 40th and 42nd day of age. Careful macroscopic examination at autopsy confirmed the complete removal of thyroid tissue in the totally thyroidectomized animals. In the partially thyroidectomized birds some regeneration of thyroid tissue was observed but in no case was the total amount of thyroid tissue in operated animals equal to that of non-operated controls.

It is obvious from table 2 that normal comb growth is dependent upon an adequate amount of thyroid hormone. The comb growth was remarkably reduced by total thyroidectomy while in the partially-thyroidectomized birds the reduction in comb size was still evident but to a less striking degree. The lower gonad weights in the experimental series suggested that the observed effect may have been mediated through a reduced gonad activity. On the other hand, it seemed pertinent that in the partially-thyroidectomized birds a definite inhibition of comb growth was observed with only a moderate change in relative gonad weight (Table 2). The absolute gonad weights, however, were noticeably lower in this

series so it is difficult to assess the activity of the gonads from these data alone.

(3) Effect of thyroidectomy and castration

A direct influence of the thyroids on comb growth was revealed by a study of thyroidectomized capons (Table 2). It has been well established that castration results in atrophy of the secondary sex characters. This was indeed the case in 40-day-old White Leghorns capons. A still further reduction in comb growth was noted, however, when thyroids as well as gonads were removed. Simmons (1943) reported similar results in his investigations on the same variety of birds. He was able to show an augmentation of comb growth in capons proportional to the amount of thyroxine injected and concluded that the amount of thyroid hormone

TABLE 3. EFFECT OF TESTOSTERONE PROPIONATE ON GONAD AND COMB WEIGHTS IN THYROIDECTOMIZED BIRDS. (40 DAYS OLD)

Treatment	No.	Body gms.	Gonads mgs.	Comb mgs.
Control (Sesame oil)	11	360	36.66	653
T.P. in oil	25	355	15.40†	1264*†‡§
T.P. with subtotal thyroidectomy	9	306	16.49†	784
T.P. with total thyroidectomy	4	223	13.89†	448‡

All gland and comb weights are expressed as milligrams per 100 gms. body weight.
*† Difference from control significant at the 5% and 1% level respectively.
‡ Difference from T.P.—subtotal thyroidectomy series significant at the 1% level.
§ Difference from the T.P.—total thyroidectomy series significant at the 5% level.

available to condition or augment the male hormone "plays an important part in the growth of the comb."

(4) Effect of testosterone propionate in thyroidectomized birds

Injection of 50 gamma of testosterone propionate daily from the 10th to the 39th day into normal, partially thyroidectomized and totally thyroidectomized birds reduced the mean relative weight of the gonad in all cases studied (Table 3). This was probably due to the inhibiting effect of the androgen on the gonadotrophic activity of the pituitary. These gonads probably contributed very little to the growth of the comb. It is obvious from table 3 that the effect of the administered androgen in these birds was directly related to the amount of thyroid tissue present.

(5) Effect of testosterone propionate in thiouracil-treated birds

A similar phenomenon was observed when the level of circulating thyroid hormone was lowered by the use of thiouracil. Thirty-one 10-day-old birds were placed on a 0.1% thiouracil diet and a like number of birds were continued on a regular diet. On the next day and each day thereafter up to and including the 39th day, sixteen of each group were given a 0.1 ml. injection of Sesame oil containing 50 gamma of testosterone propionate.

The remaining fifteen of each group were given injections of an equal amount of Sesame oil for the same period. The weight and comb size of each bird were determined every three or four days. Twenty-four hours after the last injection the birds were sacrificed and comb, gonad, thyroid, and body weights recorded (Table 4). Comb size was determined by measuring the height and length in millimeters, and a size factor was calculated by dividing the product of these two figures by two: $(H \times L/2)$.

As in the previous experiment, the injection of testosterone propionate caused a marked reduction in the mean relative weight of the gonads. Here again, the ability of the thyroid hormone to augment the action of the administered androgen in promoting comb growth is indicated (Table 4, figure 1).

TABLE 4. EFFECT OF TESTOSTERONE PROPIONATE ON GONAD AND COMB WEIGHTS IN THIOURACIL-TREATED BIRDS. (40 DAYS OLD)

Treatment	No.	Body gms.	Gonads mgs.	Comb mgs.
Control	9	398	35.2	530
.1% Thiouracil	8	239	32.6	94†‡
T.P.	15	403	20.6†	916*‡
T.P. and .1% Thiouracil	11	267	22.5§	322

Comb and gland weights are expressed as milligrams per 100 grams body weight.
*† Difference from control series significant at the 5% and 1% level respectively.
‡ Difference from the T.P.—Thiouracil series significant at the 1% level.
§ Range was from 10.3 to 67.6 with the second highest weight 28.4 mgs. This probably accounts for the lack of statistical difference from the control series.

Further analysis of the data suggests that the relationship between androgen and thyroid hormone is a synergistic one and not merely an additive phenomenon. The mean relative comb weight in capons (128 mgs.) at 40 days of age represents non-androgenic growth and can be considered as due in large part to the thyroid hormone and non-hormonal factors. It seems justifiable, also, to assume that the 322 mgs., relative weight, induced by the testosterone injections in thiouracil-treated birds represents an amount of growth due mostly to the injected androgen. On an additive basis then, when the thyroids are functioning normally and a similar dose of testosterone is given, one might expect a comb growth of about 450 mgs., relative weight. As indicated above, such a treatment actually induced a comb growth of 1,264 mgs. per 100 gms. body weight, over the period of treatment. Again, if one adds the effect of testosterone in a thyroidectomized bird (448 mgs.) to the amount of non-androgenic comb growth (128 mgs.) a total growth of 576 mgs. would be expected if the two hormones were acting simultaneously on the comb in an additive manner. In either case, it seems clear that the effect of testosterone pro-

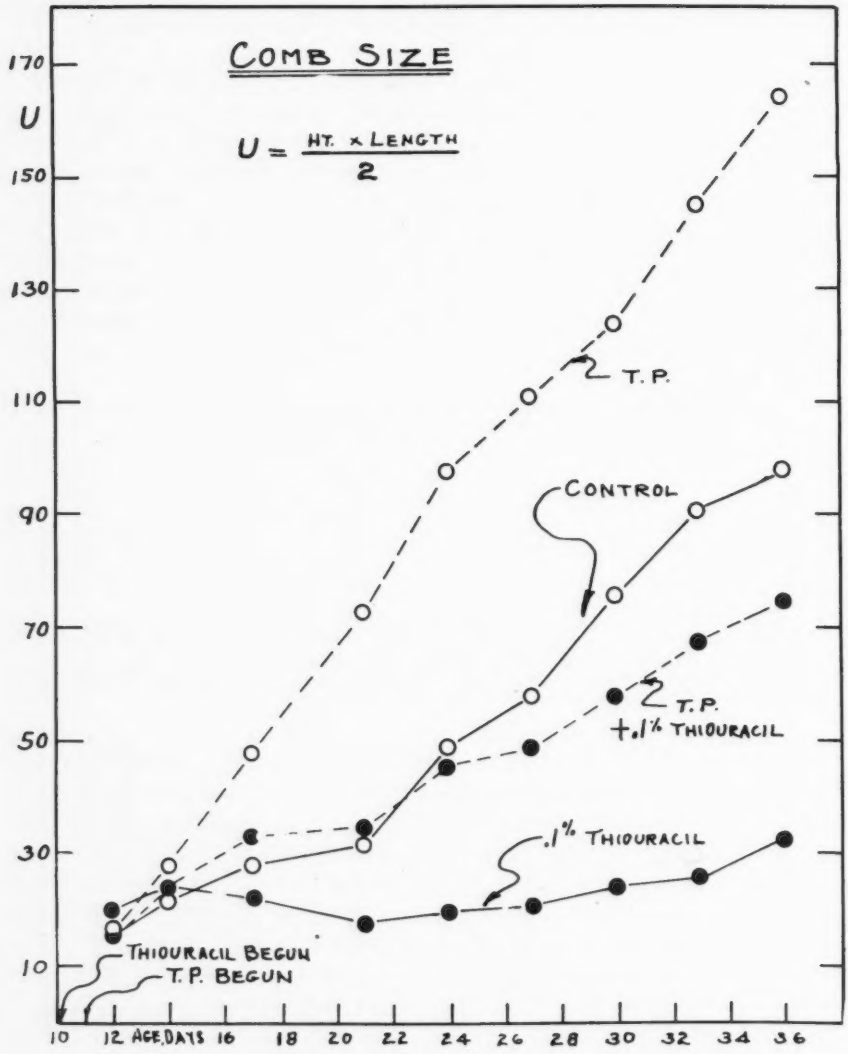

FIG. 1. Comb growth as influenced by thiouracil and testosterone propionate, together and separately.

pionate on comb growth in the presence of the thyroid gland was much greater than can be accounted for if no synergistic relationship is postulated.

SUMMARY

Data are presented to support the proposal of Simmons (1943) that comb growth in the White Leghorn cockerel is facilitated by the thyroid hormone. While the retarded comb growth in birds kept on a quantitatively restricted diet can be correlated with reduced gonad activity, the reduced thyroid weight in these birds suggests that a lowered thyroid activity might also play a part in this phenomenon. Lowering the level of circulating thyroid hormone by subtotal and total thyroidectomy showed the comb growth to be related to the amount of thyroid tissue present. Injection of large doses of testosterone propionate, sufficient to double the size of the comb of normal birds, was considerably less effective in hypothyroidic birds, which still showed a comb growth much reduced over that of the normal chicks. Removal of the gonads retarded the comb growth but a still further reduction was obtained by thyroidectomy of capons.

Evidence is presented to show that the relationship between the thyroid hormone and androgen is a synergistic one. This is supported by an analysis of data from experiments on androgen-injected thyroidectomized and thiouracil-treated birds and a study of comb growth in thyroidectomized capons.

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THE INFLUENCE OF OVARIAN HORMONES ON THE ENZYMIC ACTIVITIES OF TISSUES*

RUTH SLATON HARRIS** AND SAUL L. COHEN

From the Department of Physiological Chemistry and the Max H. Hoffman Endocrinological Research Laboratory, University of Minnesota Medical School, Minneapolis, Minnesota

A NUMBER of years ago Fishman (1944, 1947a) reported that ovariectomy in the mouse resulted in a marked decrease in the β-glucuronidase activity of the uterus and that this effect could be reversed by subsequent treatment of the animals with estrogens. On the basis of these observations Fishman proposed that the β-glucuronidase of the uterus was concerned with the metabolism of estrin in this organ and suggested that the enzyme might be involved in "metabolic conjugation" of this hormone. Kerr, Levvy and Campbell (1947) reported a relationship between mitosis and glucuronidase activity in a wide variety of tissues and suggested that the glucuronidase activity of a tissue was a reflection of growth in that tissue. In accord with this theory Levvy, Kerr and Campbell (1948) have attributed the increased β-glucuronidase activity in the liver, kidney and spleen of animals fed menthol or borneol (Fishman, 1940) as associated with growth changes following the damaging effects of these agents.

With the exception of a few observations of Fishman (1947) on the vagina, in the experiments reported by the above authors the uterus was the sole sexual tissue examined. No reports on simultaneous effects on the activities of other enzymes of procedures which modify the glucuronidase activity of tissues have appeared. The experiments reported in this paper were undertaken to supply information on these points. The activities of β-glucuronidase, alkaline phosphatase and of esterase have been simultaneously studied in the uterus, vagina, kidney, liver and spleen. Hormonal variations whose effects on these enzymes have been studied include ovariectomy, estrone injections in varying concentrations and estrone plus progesterone administration.

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EXPERIMENTAL

METHODS

Preparation of Tissues. Female mice of the Wistar stock were used in these experiments. Ovariectomies were performed when the animals were about 60 days old, and both intact and operated animals were used for enzyme studies when about 90 days old. At this time the animals were killed by ether anaesthesia, the tissues to be studied rapidly removed, chilled and weighed. In practically all cases the corresponding tissues from three mice were pooled prior to assay; the vaginae of the untreated ovariectomized mice were so small that these were frequently pooled in groups of six in order to obtain enough tissue for all the assays.

Assay Methods. The β-glucuronidase was assayed by the method of Fishman, Springer and Brunetti (1948).

The alkaline phosphatase was determined by the method of Greenstein (1941) modified in that the standard curve was prepared using the more easily weighed tyrosine rather than phenol (Folin and Ciocalteu, 1927).

The esterase activity of the tissues was determined by the method reported by Glick (1934) for tissue section studies and modified by us to be applicable to larger masses of tissues (reagents and solvents were employed in volumes 150 times as great as those used by Glick).

Control blanks and duplicate assays were run for most determinations, and the results calculated as averages of the duplicates. One enzyme unit refers to the liberation of one gamma per hour incubation of phenolphthalin, phenol, and butyric acid by the glucuronidase, alkaline phosphatase and esterase respectively.

Since estrogens are known to have a marked effect on the water content of the uterus (e.g. Astwood, 1938) nitrogen assays were also frequently carried out on the tissues. This permitted for the calculation of the enzyme activities in terms of either a tissue wet weight or a tissue nitrogen basis. The values for uterus and vagina were within a narrower range when calculated on a tissue nitrogen basis than on a wet weight basis and on occasion the enzyme activities/mg. tissue N showed effects which were masked when calculated on a wet weight basis. Consequently all values for hormonal effects on enzyme activities of the uterus and vagina are reported in terms of units per mg. tissue nitrogen. On the other hand no significant differences were shown by the spleen, liver and kidney when enzyme concentrations were calculated by the two methods and subsequently the values reported for these tissues is on a wet weight basis. It was thus possible to utilize the maximum number of assay values involved in these studies.

Hormone Administration. Estrone in 8% alcohol was administered subcutaneously twice daily for 3 days. On the 4th day the mice were sacrificed, the tissues removed and assayed. The total amounts of estrone used over the 3 days for different groups of animals were 1γ, 10γ and 100γ per mouse. The concentration of all hormone solutions were adjusted so that a volume of 0.2 cc. was injected each time.

In one group of ovariectomized mice estrone in 8% alcohol was administered twice daily for 3 days. On the latter 2 days and on one additional day progesterone was similarly administered. On the 5th day the animals were killed and the tissues assayed for their enzymic activities. In this experiment the total progesterone dose was 100γ and the total estrone dose 10γ per mouse.

A number of control experiments indicated that the injection of 8% alcohol alone had no effect on the enzymic activities of the tissues examined.

RESULTS

1. *Effect of Ovariectomy.* The enzymic activity of tissues in normal and ovariectomized mice are shown in Table 1. About 45 mice were involved in the assays for each group. Castration effected changes in enzyme activities only in the uterus and vagina, with no significant alterations being observed for kidney, liver and spleen. It is seen that ovariectomy effected a marked decrease (of about 50%) in the β-glucuronidase activity of the uterus, with no effect on that of the vagina. The castrated animals also showed reductions in the alkaline phosphatase content of both the uterus and vagina (30% and 55% respectively). Finally ovariectomy resulted in a marked increase in the esterase activity of the uterus and vagina (87% and 190% respectively). It can be seen from Table 2 that similar though quantitatively somewhat different changes are observed when the enzyme

TABLE 1. EFFECT OF OVARIECTOMY ON ENZYME ACTIVITIES OF TISSUES

Tissue	Av. weight of tissue mg.		Units enzyme/mg. wet weight of tissue					
			Glucuronidase		Alkaline Phosphatase		Esterase	
	Normals	Ovect.	Normal	Ovariec-tomized	Normal	Ovariec-tomized	Normal	Ovariec-tomized
Uterus	41	8.3	3.1±0.18*	1.6±0.31	23.2±3.3	15.7±2.0	43.5± 5.25	81.3± 8.56
Vagina	36	9	1.6±0.22	1.6±0.37	15±1.8	6.8±1.2	29.2± 4.34	85.4± 6.65
Kidney	294	321	1.1±0.08	1.0±0.09	129±9.8	121±8.8	290±18.4	308±14.9
Liver	1250	1360	1.8±0.35	1.7±0.23	—	—	1070±149	910±113
Spleen	139	151	6.0±0.4	7.0±0.61	4.6±0.54	4.8±0.54	65.2± 9.6	59.6± 2.53

* All enzyme assay results are expressed as averages ± standard error.

activities are calculated on the basis of tissue nitrogen rather than on the basis of wet weight.

2. *Effect of the Administration of Estrone.* The effects of the administration 1, 10 and 100, estrone, divided into 6 doses and administered over 3 days, on the enzymic activities of both normal and ovariectomized mice is shown in Table 2. It is readily seen that all of the enzyme changes effected by ovariectomy are reversed by the estrone administration. With the exception of esterase activities these changes were complete for even the lowest estrone dose tried (1γ). Thus in the castrated animals estrone effects a rise in the β-glucuronidase content of the uterus, an increase in the alkaline phosphatase and a drop in the esterase activities of both the uterus and vagina.

Estrone seemed to have little effect on the uterine and vaginal enzyme activities of intact animals. We are inclined to consider the just significant increases in the activities of all 3 enzymes over the normal levels effected by the administration of 10γ estrone to the normal animals of little importance since the values for this group of untreated animals calculated on a wet weight basis were amongst the lowest of those observed in the much larger group of animals assayed for the data shown in Table 1.

TABLE 2. EFFECT OF ADMINISTRATION OF ESTRONE ON TISSUE ENZYME ACTIVITIES OF NORMAL AND OVARIECTOMIZED MICE

Tissue	Total amount estrone given γ	Average weight of organ mg.		Units enzyme/mg. tissue N					
				Glucuronidase		Alkaline Phosphatase		Esterase	
		Normal	Ov'ect.	Normal	Ov'ect.	Normal	Ov'ect.	Normal	Ov'ect.
Uterus	0	41 (12)*	8.3 (9)	148 ± 32	99 ± 11.7	724 ± 194	414 ± 14.5	2230 ± 288	4560 ± 475
	1		32.4 (12)		167 ± 22.6		1168 ± 238		4240 ± 537
	10	71.4 (12)	37.9 (20)	202 ± 14.7	156 ± 16	1590 ± 338	1064 ± 248	3460 ± 370	4230 ± 482
	100	66.7 (9)	47.5 (18)	170 ± 19	188 ± 40	1353 ± 471	4548 ± 471	3330 ± 498	3253 ± 281
Vagina	0	36.2 (12)	9.2 (9)	169 ± 16.7	176 ± 39	1650 ± 364	531.7 ± 153	3210 ± 201	5670 ± 1830
	1		39.2 (18)		101 ± .89		1815 ± 524		4840 ± 1750
	10	50.8 (12)	39.6 (24)	145 ± 20.2	142 ± 34.3	1520 ± 655	1920 ± 316	3190 ± 573	4840 ± 1500
	100	47 (9)	49.7 (18)	112**	125 ± 18.6	2100	3440 ± 695	2550 ± 619	1680 ± 354
				Units enzyme/mg. tissue (wet weight)					
Kidney	0	294 (45)	321 (45)	1.1 ± 0.08	1.0 ± 0.09	128 ± 9.8	121 ± 8.8	290 ± 18.4	308 ± 14.9
	10	316 (9)	306 (15)	0.6 ± 0.09	1.1 ± 0.22	109 ± 10.8	94 ± 19	303 ± 21.5	278 ± 7.9
	100	325 (9)	321 (18)	0.8 ± 0.05	0.9 ± 0.03	78 ± 19.1	117 ± 13	309**	272 ± 20.8
Liver	0	1250 (45)	1360 (45)	1.8 ± 0.35	1.7 ± 0.23	—	—	1070 ± 149	910 ± 113
	10	1340 (9)	1340 (15)	1.5 ± 0.17	1.9 ± 0.69	—	—	1140 ± 168	760 ± 163
	100	1355 (9)	1390 (18)	1.5 ± 0.32	1.3 ± 0.22	—	—	1217 ± 152	940 ± 207
Spleen	0	139 (45)	151 (45)	6.0 ± 0.4	7.0 ± 0.61	4.6 ± 0.54	4.8 ± 0.54	65.2 ± 9.6	59.6 ± 2.53
	10	173 (12)	140 (15)	6.0 ± 0.73	7.0 ± 1.17	4.9 ± 1.37	4.2 ± 0.66	63.8 ± 3.2	50.5 ± 4.12
	100	155 (9)	175 (15)	5.0 ± 0.79	4.9 ± 0.52	5.3 ± 0.46	4.9 ± 1.63	64.7 ± 10.1	65.2 ± 9.2

* Figures in parenthesis indicate the total number of animals involved in each group.
** Because of an accident only 2 assay values were obtained in this group, and therefore only the average for these values is given.

Increasing the estrogen dosage to the ovariectomized animals seems in several instances to carry the enzyme changes beyond merely the restorative stage. Thus in the 100γ treated animals the alkaline phosphatase of both uterus and vagina showed a 5–10 fold increase over that of the untreated ovariectomized animals and a 2–3 fold increase over the activities of similarly treated intact animals. The esterase in the vagina only of the 100γ estrone treated ovariectomized animals showed a significantly lower (about 50%) esterase content than that of similarly treated intact animals.

Some though less marked effects on the enzyme activities of the kidney, liver and spleen were observed. Thus there seemed to be an indication of a reduction in the activities of all 3 enzymes in the kidney and in the

TABLE 3. EFFECT OF PROGESTERONE AND ESTRONE ON ENZYME ACTIVITIES OF TISSUES OF OVARIECTOMIZED MICE

Tissue	Average weight of tissue mg.		Units enzyme/mg. tissue N					
			Glucuronidase		Alkaline Phosphatase		Esterase	
	E*	E+P	E	E+P	E	E+P	E	E+P
Uterus	37.9 (20)	24.5 (18)	156±16	116±34.1	1064±248	847±230	4230±482	4600±488
Vagina	39.6 (24)	28 (15)	142±34.3	61± 3.5	1920±31.6	1620±266	4840±1500	3100±800
			Units enzyme/mg. tissue (wet weight)					
Kidney	306 (15)	320 (12)	1.1± 0.22	0.38± 0.04	93.5±19	101.9±16.7	278±7.9	360±49
Liver	1340 (15)	1175 (12)	1.9± 0.69	0.75± 0.13	—	—	760±163	1704±44.5
Spleen	140 (15)	127 (9)	7.0± 1.17	2.7 ± 0.28	4.18± 0.66	5.6± 0.77	50.5±4.12	93.8±20.4

* E = Animals treated with 10γ estrone.
E+P = Animals treated with 10γ estrone and 100γ progesterone.

glucuronidase of the liver. The changes were, however, for the most part relatively small and are not considered worthy of further discussion unless substantiated by assays on much larger groups of animals than those used in the studies reported here.

3. *Effect of Progesterone.* The tissue enzyme activities of ovariectomized mice treated with 10γ estrone plus 100γ progesterone together with those of the animals treated with 10γ estrone alone are shown in Table 3. The progesterone caused a marked drop (average 60%) in the glucuronidase activity of the vagina, kidney, liver and spleen. A lesser and statistically questionable reduction in the glucuronidase content of the uterus resulted from progesterone administration. The only other significant changes resulting from the progesterone was an increase in the esterase activities of the liver and spleen.

DISCUSSION

1. *Effects of ovarian hormones on glucuronidase of tissues.* Our findings on the effect of ovariectomy and of estrone administration on the glucuronidase activity of the uterus are consistent with those previously reported

by other workers (Fishman, 1947, Kerr *et al.*, 1950). Fishman reported that in some cases the vaginae of ovariectomized animals also showed an increase in the glucuronidase activity in response to estrogen. Our experiments showed no change in the glucuronidase activity in the mouse vagina following either ovariectomy or estrone administration. Since both the uterus and vagina of the ovariectomized animals show a marked morphological response to estrogen therapy the failure of the vagina to show corresponding glucuronidase changes must mean that if β-glucuronidase is involved in the metabolism of the estrogenic hormone as suggested by Fishman then this involvement is not demonstrated for the vagina in these studies. The failure of the vagina to show changes in β-glucuronidase activity even though the estrone administration causes a marked increase in the size of the vagina of the ovariectomized animal is likewise difficult to reconcile with the hypothesis proposed by Kerr *et al.* in which the glucuronidase activity of a tissue is related to its degree of growth.

It is possible that a decreased β-glucuronidase content in the vagina of the castrated mouse may be at least partially masked by a relatively high glucuronidase activity of the leukocytes (Fishman *et al.*, 1948) which are present in large numbers in the vaginal epithelium of the castrated animal. Lacking precise data, however, on the glucuronidase content of the polymorphonuclear leukocytes and on the proportion of these in the castrate vagina the significance of such a possibility cannot be assessed.

The finding of Kerr and coworkers that the livers of ovariectomized mice showed an increased glucuronidase activity in response to the administration of estrone (1949, 1950) was not confirmed in our experiments.

Both Fishman and Kerr and coworkers have reported that progesterone alone administered to ovariectomized animals effected no change in the glucuronidase activity of the uterus. This is readily explained on the basis of a relative insensitivity of the uterus to progesterone in non-estrogenized animals. Kerr and coworkers (1950) have, however, reported that progesterone antagonizes the effect of estrone on the glucuronidase response of the uterus of ovariectomized mice. In our data this change, if significant, was relatively small. A much greater inhibition of progesterone on the glucuronidase activity of the other tissues examined, namely, the vagina, kidney, liver and spleen, was noted. Since the progesterone effected a greater inhibition in the growth response of the uterus than for any other tissue (the changes being insignificant for the kidney, liver and spleen), our observations with progesterone are thus impossible to explain by the "growth" theory advanced by Kerr *et al.* for β-glucuronidase significance.

The differences in the observations reported by Kerr *et al.* and those reported in our own studies may be due to differences in one or more of the following:

1. A difference in the response of different strains of animals. This suggestion is lent support by the recent report of Morrow, Greenspan and Carroll (1949) indicating a marked difference in the glucuronidase activities of the livers of different strains of mice.

2. A difference in the mode of administration and in relative amounts of hormones administered. In our experiments the hormones were administered in 8% alcohol in divided dosage over 3 days. Kerr and coworkers administered their hormones in oil and by a single injection. While the amount of estrone used in both sets of experiments was about the same, the progesterone/estrone ratio used by them was approximately 1:1 while in our experiments the ratio employed was 10:1.

3. The use of different assay methods for the determination of glucuronidase activity. The main difference lies in the nature of the substrate, which in our experiments was phenolphthalein glucuronide, while Kerr *et al.* employed phenylglucuronide.

Whether any of these differences in procedure can account for the observed differences in results in our data as compared to those of Kerr *et al.* can only be determined by further experimentation.

2. *Effects of ovarian hormones on the alkaline phosphatase of tissues.* Our experiments indicated a more uniform relationship between alkaline phosphatase activity of tissues and growth than were observed for β-glucuronidase. Our failure to obtain significant alkaline phosphatase assays for liver in any of our animals may be due to the lack of magnesium in our assay mixture since Cloetens (1939) showed that alkaline phosphatase of the liver requires magnesium activation for activity more than does that of anv other tissue.

Using histochemical techniques Atkinson and Elftman (1947) have reported an increase in the alkaline phosphatase of the uterus in the ovariectomized mouse following the administration of estradiol dipropionate. They suggest that this change may be associated with one or more of the following effects of estrogen on the uterus: (a) lipid disappearance, (b) increased glycogen content, and (c) increased protein synthesis (see also Atkinson and Engle, 1947). Our quantitative determinations on the alkaline phosphatase content of the uterus confirms their observations and also indicates that similar changes occur in the vagina, namely a decreased alkaline phosphatase content resulting from ovariectomy and an increase following the administration of estrone to ovariectomized animals. Since we have no data on the effect of estrogens on the lipid or glycogen content of the vagina the possible involvement of these factors in the alkaline phosphatase activity of the vagina cannot be assessed. The degree of protein anabolism, however, might well be involved in the

alkaline phosphatase changes observed for both the uterus and vagina.

3. *Effects of ovarian hormones on the esterase of tissues.* A search of the literature has not revealed to the authors any previous work on the relationship of the sex hormones and the esterase activity of tissues. Our data indicate a marked increase in the esterase activity of both the uterus and vagina following castration and a restoration of these on estrogen administration. In the absence of any specific information on the function/s of esterase in tissues the significance of these findings cannot be assessed. These results might be explained on the assumption of some direct relationship between the lipid content of a tissue and its esterase activity. On this basis one would have to assume also that estrone plus progesterone caused increases in liver and spleen fat. These possibilities are to be tested, but pending further data the observations on esterase reported in this paper must be considered as unexplained.

In most cases the administration of either 1 μg. or 10 μg. estrone to ovariectomized mice effected restorative changes in the enzymic activities of the uterus and vagina and had no effect on the enzymic activity of these tissues in the intact animal. While 100 μg. estrone had little effect on any of the enzymic activities studied in intact animals, marked changes beyond restorative effects occurred for uterine and vaginal alkaline phosphatase and for vaginal esterase in ovariectomized animals. These results indicate that the presence of the ovaries tend to protect the uterus and vagina from the effects of large doses of estrogen.

SUMMARY

1. Observations on the effect of ovariectomy, of estrone and of estrone plus progesterone administration on the β-glucuronidase, alkaline phosphatase and esterase activities of tissues in the mouse have been reported. The tissues studied included 2 sexual tissues—uterus and vagina,—and three non-sexual tissues,—kidney, liver and spleen.

2. Ovariectomy causes a marked drop in the glucuronidase activity of the uterus only and significant decreases in the alkaline phosphatase and increases in the esterase activities of both the uterus and vagina.

3. The effects of ovariectomy were reversed by the administration of estrone.

4. Progesterone administered with estrone causes a marked reduction in the β-glucuronidase activity of the vagina, kidney, liver and spleen and an apparent increase in the esterase activity of the liver and spleen.

5. The presence of the ovaries appears to stabilize the response of the uterus and vagina to large doses of estrone.

6. Certain possible significances of these data are discussed.

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THE EFFECT OF INDUCED OVULATION ON PREGNANCY

H. O. BURDICK AND MALCOLM CRUMP[1]

From the Department of Biology, Alfred University, Alfred, New York

INVESTIGATORS have presented conflicting reports regarding the effect of induced ovulation on pregnancy in the mouse, rat, and rabbit. In 1928, Engle and Mermod stated that ovulation did not occur unless the pregnancy had just terminated. In that same year, Zondek and Aschheim reported the presence of living fetuses in one pregnant mouse which had ovulated after several injections of chorionic gonadotrophin. Later, Snyder and Wislocki (1931) observed living fetuses in two rabbits which had ovulated. After inducing ovulation in several other pregnant rabbits, however, they found that the fetuses were aborted or resorbed. Bellerby (1935) reported abortion or resorption after induced ovulation in rabbits in the third trimester. Snyder and Coteen (1939) caused abortion in rabbits on the seventeenth day of pregnancy by injecting 10 R.U. of pregnancy urine. A similar injection on the eleventh day did not result in abortion. Burdick *et al.* (1943) showed that ovulation was induced in the pregnant mouse by a smaller single injection of chorionic gonadotrophin than was necessary to produce the same result in the diestrous mouse. The effect of induced ovulation on the further course of pregnancy could not be observed, however, because the autopsies were performed approximately twenty hours after the single injection.

The present investigators thought that the lack of uniformity in the results of these earlier experiments might have been due to such factors as differences in extracts, amounts used, or repeated injections, rather than to induced ovulation *per se*. It was decided, therefore, to determine whether pregnancy was affected by ovulation induced by a single injection of a chorionic gonadotrophin of predetermined potency.

PROCEDURE

Female albino mice of the Rockland strain were selected after the animals had mated and the vaginal plug had formed. At varying stages of pregnancy, these animals were given one subcutaneous injection of the gonadotrophin to induce ovulation. The minimal ovulating dose of this particular chorionic gonadotrophin[2] had been previously found

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[1] Present address: Albany Medical College, Albany, New York.

[2] Generous supplies of the chorionic gonadotrophin "APL" were furnished by Ayerst, McKenna and Harrison Ltd., Rouses Point, New York.

to be 1.5 I.U. and the dosage varied from 1 I.U. to 5 I.U. Approximately twenty hours after the injection, each mouse was anesthetized with sodium nembutal (0.075 gr.) and prepared for laparotomy. The left ovary was exposed by a small dorsal incision and the ampulla was examined under a dissecting microscope, after the anterior end of the reproductive tract had been gently pulled through the incision. A distended ampulla indicated ovulation. Frequently, ova with granulosa cells could be seen within the thin-walled ampulla. After the tract had been replaced and the incision sutured, each animal was placed in a separate cage and inspected daily throughout the gestation period for vaginal bleeding indicative of abortion. After parturition, the size and condition of the young were noted.

RESULTS

About thirty pregnant mice not shown in Table 1 were used, either to establish the minimal ovulating dose or to serve as controls. Animals

TABLE 1. EFFECT OF INDUCED OVULATION ON PREGNANCY

Animal number	Dosage in I.U.	Day of preg. injected	Ovulation	Number of young at term	Size of young	Condition of young
15	3	4	+	11	0.5 cm.	10 day fetuses examined in utero with beating hearts
37	3	9	+	7	2.7	Living
36	2	11	+	7	3	Living
35	2	12	+	9	3	Living
42	3	11	+	5	3	Living
55	1.5	12	+	9	3	Living
43	3	12	+	12	3	Living
47	3	12	+	9	3	Living
34	2.5	13	+	10	3	Living
25	3	13	+	9	3	Living
44	3	13	+	9	3	Living
5	1	16	+	7	3	Living
33	2.5	16	+	7	3	Living
38	3	16	+	7	2.7	Living
40	5	14	No Laparotomy	7	3	Living
46	5	17	No Laparotomy	7	3	Living
41	5	17	No Laparotomy	6	3	Living
39	5	17	No Laparotomy	4	3	2 living 2 killed

injected with sub-threshold amounts of gonadotrophin are not included in the Table.

Only one mouse was injected during the first third of pregnancy. This animal received 3 I.U. of the gonadotrophin four days after the mating and showed ova in the ampulla twenty hours later. There was no sign of pregnancy failure at the autopsy on the tenth day after mating, and when the fetuses were removed from their amnionic cavities, the heart of each was beating normally. The size and appearance of the fetuses were normal for that period in development.

Seven animals were injected during the second period of pregnancy (9

to 13 days) with doses ranging from 1.5 I.U. to 3 I.U. All animals were checked for ovulation and then allowed to go to term. Parturition was apparently normal and the litters varied from seven to twelve in number. With only one exception, the young measured 3 cm. in length, which is normal for this period.

In the third period of gestation, six animals ovulated after injections of 1. I.U. to 3 I.U. Their litters were normal in number and size. Laparotomies were not performed on four other mice which had each received 5. I. U. of the gonadotrophin and allowed to go to term. These also had normal litters. One mouse (No. 39) had only four young, two living and two dead. It is possible that this female may have disposed of some of the young before her cage was checked.

DISCUSSION

From the above results, it is evident that ovulation can be induced in the pregnant mouse without interfering with the continued development of the fetuses. In view of this observation, it seems safe to assume that in some of the earlier experiments, abortion and resorption of fetuses must not have been due to ovulation, *per se*, but to some other factor such as was mentioned earlier in this report.

The relative ease of inducing ovulation in the pregnant mouse should again be emphasized, since some investigators maintain that active corpora lutea prevent ovulation. We are inclined to agree with Wolfe (1931) that there is possibly some factor other than the corpus luteum that inhibits normal ovulation during pregnancy.

We wish also to re-emphasize the importance of determining the effects of a single injection of gonadotrophin 18 to 24 hours after the injection. Inspection of the ovaries following a series of daily injections of a gonadotrophin may not be correctly interpreted unless it is known how the ovaries responded to the first and subsequent injections. Ovarian changes in weight, follicle development, and luteinization depend, in part, on whether or not ovulation was induced early in a series of injections.

SUMMARY

A single subcutaneous injection of 1.5 I.U. to 5 I.U. of a chorionic gonadotrophin readily induced ovulation during various stages of pregnancy in the mouse. The presence of active corpora lutea of pregnancy did not inhibit ovulation under these conditions.

Pregnancy was not terminated. The gestation period was normal and the young were normal in number and size. Abortions reported by some earlier investigators must have been due, therefore, to some factor other than induced ovulation.

Attention is called to the probability that ovarian changes in weight, follicle development, and luteinization will be affected if ovulation has been induced early in a series of injections of gonadotrophins.

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INSULIN AND ADRENOCORTICOTROPIN LABELED WITH RADIO-IODINE*

J. W. FERREBEE, M.D., B. B. JOHNSON, M.D.,† J. C. MITHOEFER, M.D. AND J. W. GARDELLA, M.D.

Mary Imogene Bassett Hospital,‡ Cooperstown, New York

INTRODUCTION

UNDER appropriate conditions iodine attaches itself firmly to the tyrosine groups in many proteins. The effect of this attachment upon the specific immunologic or hormonal activity of the protein appears to vary with the number (Wormall, 1930; Pressman and Sternberger, 1950) and perhaps with the position (Pressman and Sternberger, 1950) of the groups affected. A few atoms of iodine or radio-iodine per molecule of protein are quite compatible with the maintenance of immunologic reactivity in the case of anti-pneumococcus globulin, Figure 1. It has therefore seemed possible that a similar iodination, sufficient for radio-tracer experimentation, may not adversely affect the activity of protein hormones The purpose of this communication is to describe observations made on insulin and ACTH

METHOD OF IODINATION

Essentially similar methods of iodination were used for preparations of ACTH,[1] insulin,[2] and gamma globulins. 10 mg. of hormone were dissolved in a few milliliters of M/15 phosphate buffer pH 7.4, placed in a cold bath, and a mixture of potassium iodide, iodine, and I^{131} was added dropwise with mechanical stirring over a period of an hour or more. The strengths of the reagents used were of the order of 0.38 mg. of sodium iodide, 0.16 mg. of iodine, and 5 to 50 millicuries of I^{131} for 10 milligrams of hormone. Solutions were dialyzed free of inorganic iodide and made up to volume, or the protein with its organically bound I^{127} and I^{131} was precipitated iso-electrically or by alcohol in the cold, the precipitate washed, made up to volume, and nitrogen and radioactivity determined. Calculations based upon the amount of carrier iodine used and the amount of protein-bound radioactivity obtained indicate that 30 to 50 per cent of the iodine in valence state suitable for reaction (Li, 1945) combined with protein at pH 7.4. Approximately 2 molecules

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‡ Affiliated with Columbia University.

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[2] Obtained through the kindness of Dr. Edward Campbell, Eli Lilly Company. Indianapolis, Indiana.

of iodine per molecule of insulin were so bound in the preparations studied. Radioactivity of the order of 50–500 microcuries per milligram of hormone was present in the iodinated insulin and ACTH samples immediately following preparation depending upon the amount of I^{131} used.

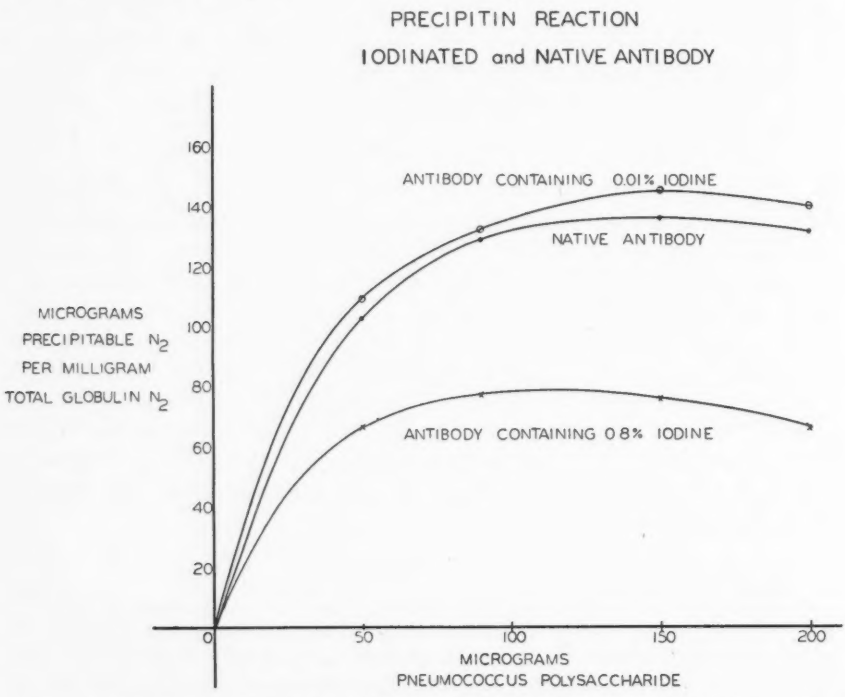

FIG. 1. Solutions contained identical amounts of globulin at identical volumes. The radioactive globulins containing 0.01 milligrams iodine per 100 mg. protein gave the same amount of precipitable antibody nitrogen as untreated globulins. The radioactive globulin labeled with about 100 times as much iodine showed a marked loss in precipitable antibody, especially in the excess antigen zone. The radioactivity per milligram of nitrogen for the entire gamma globulin fraction of anti-pneumococcus serum was 30 per cent greater than the radioactivity of the immune globulin obtained from it in the specific precipitates. This observation is consistent with the suggestion that immune globulines iodinate at a rate slower than that for gamma globulins and serum proteins in general. (Pressman and Sternberger, 1950)

Bioassay of iodinated insulin

Samples of iodinated, radioactive insulin obtained by the above methods produce hypoglycemia in fasted rabbits in degree comparable to that produced by native insulin, Figure 2. Equality of hypoglycemic activity in iodinated and native preparations is illustrated in careful mouse assays

having as end point the production of convulsions in 50 per cent of the mice tested (Harrington, Scott, Culhane, Marks and Trevan, 1929). Table I gives a comparison of the results obtained at this end point level.

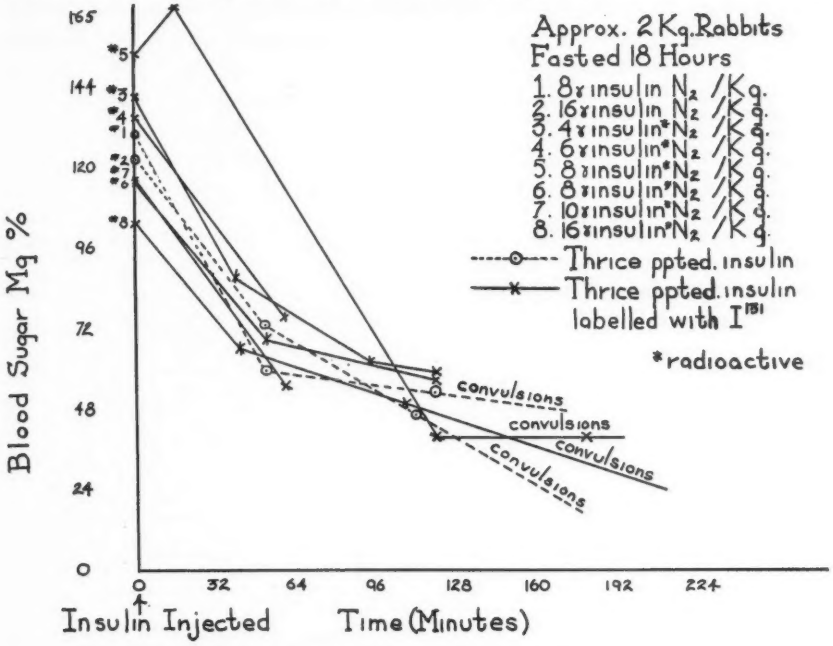

FIG. 2. Comparison of hypoglycemic effect of native and radio-labeled insulin in fasted rabbits.

Bioassay of iodinated ACTH

Preparations of ACTH were tested for hormonal activity by measuring their effect upon the concentration of ascorbic acid in the adrenals of hypophysectomized rats, using intravenous injection and one hour as test period. In general no great change in hormonal activity was occasioned by the iodination. Table II presents data obtained with a preparation of ACTH whose activity, both before and after labeling with radio-iodine, was approximately 20 times that of the standard Armour preparation LA-1A.

DISCUSSION

The data presented indicate that small amounts of radio-iodine may be attached to large molecules present in insulin and ACTH samples without evident loss of hormonal activity. Satisfactory as the evidence for unimpaired activity in these samples may be, it is nonetheless impossible by

bioassay to prove that the hormonal action is centered in the radioactive molecules. There remains within the limit of the assay method a possibility that a small percentage of heavily iodinated molecules of inconsequential biologic activity is diluted by a large percentage of uniodinated molecules. Although the method of iodination is such as to make an unequal distribu-

TABLE 1. BIOASSAY OF IODINATED AND NATIVE INSULINS IN FASTED MICE OF 25 GM. BODY WEIGHT

Insulin Preparation	Micrograms Insulin N_2 Injected	Milliliters Injected	Result
Crystalline Zinc Insulin (Lilly) Precipitated three times at pH 5.6, iodinated and dialyzed.*	0.2	0.25	Convulsions in 50 minutes
	0.2	0.25	Convulsions in 55 minutes
	0.2	0.25	Convulsions in 60 minutes
	0.2	0.25	Convulsions in 60 minutes
	0.2	0.25	Convulsions in 65 minutes
	0.2	0.25	Convulsions in 70 minutes
	0.2	0.25	Convulsions in 70 minutes
	0.2	0.25	Convulsions in 70 minutes
	0.2	0.25	No effect after 2 hours
	0.2	0.25	No effect after 2 hours
	0.2	0.25	No effect after 2 hours
	0.2	0.25	No effect after 2 hours
Crystalline Zinc Insulin (Lilly) Precipitated three times at pH 5.6	0.2	0.25	Convulsions in 60 minutes
	0.2	0.25	Convulsions in 62 minutes
	0.2	0.25	Convulsions in 65 minutes
	0.2	0.25	Convulsions in 65 minutes
	0.2	0.25	Convulsions in 75 minutes
	0.2	0.25	Convulsions in 75 minutes
	0.2	0.25	No effect after 2 hours
	0.2	0.25	No effect after 2 hours
	0.2	0.25	No effect after 2 hours
	0.2	0.25	No effect after 2 hours
5 times recrystallized beef insulin, iodinated and dialyzed.*	0.2	0.25	Convulsions in 60 minutes
	0.2	0.25	Convulsions in 67 minutes
	0.2	0.25	No effect after 2 hours
	0.2	0.25	No effect after 2 hours
5 times recrystallized beef insulin.	0.2	0.25	Convulsions in 43 minutes
	0.2	0.25	Convulsions in 63 minutes
	0.2	0.25	No effect after 2 hours
	0.2	0.25	No effect after 2 hours

* As described under iodination procedure.

tion of the iodine labeled unlikely, proof of the distribution is troublesome. In the case of immune globulin demonstration of at least the co-existence of biologic activity and radio-tracer label is made possible by specific precipitation of radioactivity through precipitin formation, Figure 1. In the case of ACTH and insulin similar demonstration requires further knowledge of the modes and sites of action of these hormones. Preliminary observations here and elsewhere (Sonenberg, Keston and Money, 1950) have been encouraging in that they have given evidence of preferential

location of radioactivity in the adrenal following intravenous injection of radioactive preparations of ACTH or following treatment of adrenal slices in vitro. Interpretation of these experiments is unfortunately complicated by problems of blood flow and mixing time in different organs and by the fact that the ACTH preparations available are by no means homogeneous. In the case of insulin data appended in Table III indicate that a firm combining ratio between insulin and some constituent of striated muscle is difficult to demonstrate (Stadie, Haugaard, Marsh and Hills, 1949). Evidence for such combination should perhaps be sought at lower concentration levels in view of observations (Willebrands, Groen, Kamminga

TABLE 2. EFFECT OF IODINATION ON ACTIVITY OF ACTH

Micrograms ACTH Nitrogen Injected i.v.	Per Cent Reduction of Adrenal Ascorbic Acid Concentration 1 Hour After Injection	
	Animals Injected with Native ACTH	Animals Injected with Iodinated ACTH
7.5×10^{-3}	11	0
7.5×10^{-3}	30	25
7.5×10^{-3}	45	27
7.5×10^{-3}	34	26
7.5×10^{-2}	57	35
7.5×10^{-2}	40	32
7.5×10^{-1}	67	52
7.5×10^{-1}	40	44

and Blickman, 1950) suggesting that the amounts of insulin necessary for demonstration of an effect upon glycogen synthesis in striated muscle are several hundred fold below those previously (Stadie and Zapp, 1947) given. Independent confirmation of this suggestion in our hands has led to a possible explanation deriving from the fact that insulin in dilute solution at pH 7 is adsorbed to a significant degree by laboratory glassware. The adsorption is demonstrated by a rapid disappearance of radioactivity from dilute solutions at pH 7 with a rapid return on the addition of strong alkali. Bovine albumin in a final concentration of 0.5 per cent prevents or delays the adsorption. Studies are therefore being undertaken to evaluate the significance of these observations with regard to the proper constitution for media used in determining the in vitro effect of insulin upon glycogen synthesis in muscle.

SUMMARY

Evidence is presented to indicate that ACTH and insulin may be labeled with radioactive iodine without loss of hormonal activity. Observations made with labeled preparations are discussed briefly.

TABLE 3. PERSISTENCE OF RADIO-INSULIN IN 475 ±75 MG. SAMPLES OF RAT DIAPHRAGM AS DETERMINED BY EXTRACTION WITH N/2 NaOH AND MEASUREMENT OF EXTRACTED RADIOACTIVITY

Individual Wash Periods	Wash Volume	Number of Washes	Micrograms of Insulin N_2 Remaining in Muscle
A. Diaphragms immersed for 1.5 minutes in 5 ml. Stadie buffer containing 50 micrograms insulin nitrogen			
30 seconds	25 ml. Stadie buffer	2	0.34
30 seconds	25 ml. Stadie buffer plus glucose	2	0.38
15 minutes	25 ml. Stadie buffer	3	0.074
15 minutes	25 ml. Stadie buffer	4	0.062
15 minutes	25 ml. Stadie buffer plus glucose	4	0.061
B. Diaphragms immersed for 1.5 minutes in 5 ml. Stadie buffer containing 5 micrograms insulin nitrogen			
30 seconds	25 ml. Stadie buffer	2	0.055
30 seconds	25 ml. Stadie buffer	2	0.053
30 seconds	25 ml. Stadie buffer	2	0.057
30 seconds	25 ml. Stadie buffer	4	0.042
30 seconds	25 ml. Stadie buffer	4	0.041
15 minutes	25 ml. Stadie buffer	3	0.014
15 minutes	25 ml. Stadie buffer	3	0.017
15 minutes	25 ml. Stadie buffer	3	0.011
15 minutes	25 ml. Stadie buffer	4	0.023
15 minutes	25 ml. Stadie buffer	4	0.025
C. Diaphragm immersed for 1.5 minutes in 5 ml. Stadie buffer containing 0.5 micrograms insulin nitrogen			
30 seconds	25 ml. Stadie buffer	2	0.0048
30 seconds	25 ml. Stadie buffer	2	0.0069
30 seconds	25 ml. Stadie buffer	4	0.0044
30 seconds	25 ml. Stadie buffer	4	0.0043
15 minutes	25 ml. Stadie buffer	3	0.002
15 minutes	25 ml.	3	0.002

TABLE 3—(*continued*)

Individual Wash Periods	Wash Volume	Number of Washes	Micrograms of Insulin N_2 Remaining in Muscle
15 minutes	Stadie buffer 25 ml. Stadie buffer	3	0.002
15 minutes	25 ml. Stadie buffer	3	0.002
15 minutes	25 ml. Stadie buffer	3	0.001
15 minutes	25 ml. Stadie buffer	4	0.001
D. Diaphragms immersed for 15 minutes in 5 ml. Stadie buffer containing 5 micrograms insulin nitrogen			
15 minutes	5 ml. Stadie buffer	3	0.044
15 minutes	5 ml. Stadie buffer	6	0.019
E. Diaphragms immersed for 15 minutes in 5 ml. Stadie buffer containing 0.5 micrograms insulin nitrogen			
15 minutes	5 ml. Stadie buffer	3	0.0042
15 minutes	5 ml. Stadie buffer	6	0.0012

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SOME FACTORS THAT INFLUENCE THE RELEASE OF IODINE FROM THE THYROID GLAND

J. WOLFF†

From the Ziskind Research Laboratories, New England Center Hospital, and the Department of Medicine, Tufts College Medical School, Boston, Massachusetts

NUMEROUS attempts have, in the past, been made to determine the rate of delivery of iodine or thyroid hormone to the circulation from the thyroid gland under varying experimental conditions. The early attempts were indirect dealing largely with thyroid histology or metabolic rate. More recently, sufficiently reliable chemical methods have become applicable to this purpose and it was possible to follow the changes in the total or thyroxine iodine in the gland (Chaney 1938, 1950; Taurog and Chaikoff, 1946a). Thus it was found that administration of thyrotropin led to marked decreases in total thyroid iodine (Loeser, 1931; Houssay *et al.*, 1932; Closs *et al.*, 1932; Grab, 1932) and in thyroxine iodine (Foster *et al.*, 1932; Chaikoff *et al.*, 1947). Indeed, it has been suggested that the iodine depletion in the thyroid gland be used as an assay for thyrotropin. Furthermore, Astwood and Bissell (1944) have shown that a similar depletion occurs in the thyroid iodine of rats treated with thiouracil. On the other hand, it has been shown that hypophysectomy fails to alter the iodine content of the thyroid (Taurog *et al.*, 1946b), and recently VanderLaan and Greer (1950) have shown this stasis to be due both to a reduction in the iodine concentrating capacity and in the rate of delivery of iodine to the circulation. These results have been confirmed by Wolff and Chaikoff (unpublished observations).

Studies on the protein-bound iodine of plasma have yielded a confirmatory picture. Thus thyrotropin can, in 24 hours, more than double the protein-bound iodine of plasma (Wolff, unpublished data), while hypophysectomy will reduce the concentration to about half (Taurog *et al.*, 1946b), as will antithyroid drugs such as thiouracil and other thiocarbonamide derivatives (Winkler *et al.*, 1946), and potassium thiocyanate when chronically administered (Wolff *et al.*, 1946).

The third line of evidence stems from studies with radioactive iodine. Here it has been possible to calculate actual turnover rates, both by the method of Hevesy and of Zilversmit (Taurog *et al.*, 1947a, b). The methods are, however, difficult to carry out due to considerable variations from

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† Present address: Harvard Medical School, Boston, Massachusetts.

animal to animal (e.g. in the per cent of the injected dose taken up by the gland etc.). In the present investigation a newer method, applying external gamma-ray counting to rats, in which case each animal serves as its own control, has been applied in a number of experimental conditions.

EXPERIMENTAL

Male Sprague-Dawley rats were kept on the following diet from the time of weaning:

Cracked Whole Wheat	66.0%
Casein	15.0%
Whole Milk Powder	15.0%
Brewer's Yeast	2.5%
$CaCO_3$ (C.P.)	1.0%
NaCl (C.P.)	0.5%

One ml. of a cod liver oil concentrate was added per kg. of diet. This yielded a supplementary ration of 50 U.S.P. units of vitamin A per gm. and 5 U.S.P. units of vitamin D per gram.

The iodine content of this diet was approximately 0.28 micrograms per gram.

When propylthiouracil was used it was added at 0.03% concentration to the above diet.

Forty hours after an intraperitoneal injection of I^{131}, and at appropriate intervals thereafter, the thyroid region of the rats were counted under constant geometrical conditions with a highly sensitive gamma tube (Sylvania GG-306) over a heavy, tapered lead shield with a narrow (18 mm.) opening (reinforced with extra lead shielding near the hole).[1] All external counting was carried out while the rats were under light ether anesthesia; this facilitated counting and the maintenance of a constant geometry.

Background for non-thyroidal tissue activity was counted over the epigastric region. This area was found to be more satisfactory than the more caudal regions of the body because of the variable amounts of I^{131} in the bladder, especially during the early intervals. This was, however, an over-correction because a larger volume of tissue was counted. Circumferences of the neck and body were measured and from the average radii thus obtained, the volumes counted were found to be in the ratio of approximate 3:1. Thus one-third of the body background was counted under the same condition as the neck, while the other two-thirds were counted at a less favorable geometry, i.e. at about 58 per cent average efficiency (50–67 per cent[2]).

Now if

N = counts over body minus absolute background

n = counts per unit volume of neck in the absence of the thyroid minus absolute background

v = volume of the neck counted

V = volume of the body counted (thus $v = 1/3V$)

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[1] Designed in 1948 by Dr. M. S. Raben of this laboratory.

[2] Three extreme points were chosen to estimate the range of efficiency: at the most distal point of the body, at the most distal point of the neck, and at the base of these. The squares of their distances from the sensitive region of the tube (i.e., including the dead space in the tube) were found to be in the ratio of 2:1.5:1 and hence the range of 1/2 to 1/1.5 or 50 to 67 per cent.

Then

$$1/3Vn + 2/3V\frac{58}{100}n = N$$

and

$$n = \frac{N}{0.72V} = 1.39\frac{N}{V}$$

and the background over the neck is

$$nv = 1.39\frac{Nv}{V} = \frac{1.39N}{3} = 0.46N$$

and the background over the neck is thus $\approx N/2$.[3]

These approximations were checked experimentally by counting the necks of 19 animals after removal of the thyroids with values of N ranging from 0.5 to 33.4 counts per second. The background over the neck was found to be $nv = (0.51 \pm 0.027[4])N$ or again $\approx N/2$. Thus in the calculations of the results presented here the total count over the thyroid was corrected by one half the body background as well as the absolute background.

Furthermore, in order to reduce the external count of all of the animals to the same scale, the *initial or zero time count was considered as* **100** *per cent* of thyroidal I^{131} and all subsequent counts were expressed as percentages thereof.

RESULTS

1. *Normal and Propylthiouracil-Treated Controls.* The rats were injected with 30–35 microcuries of I^{131} by the intraperitoneal route 40 hours before "zero time." At this time the first external count was made and it was found that from 12.6 to 41.2 per cent of the injected dose had been taken up by the thyroid gland. *These values at zero time were taken as 100 per cent* and all subsequent determinations are expressed in terms of this initial value for each individual rat.

When propylthiouracil treatment was instituted it was started immediately after the zero time count by an intraperitoneal injection of 10 mg. of propylthiouracil suspended in saline.

The decreases in I^{131} of the thyroids of normal and propylthiouracil-treated rats, which served as controls for many of the subsequent experiments are graphically represented in Figure 1 and are listed with standard errors in Table 1. The values were corrected for decay of the isotope. It is

[3] If changes in efficiency with changes in distances had not been accounted for then:

$$nv = N\frac{v}{V} = \frac{N}{3}.$$

[4] Standard error of the mean.

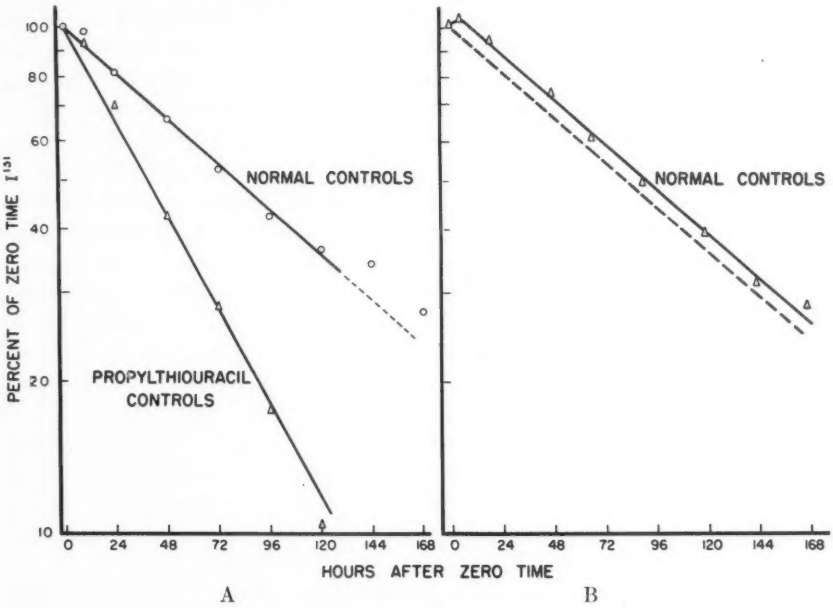

FIG. 1A. The release of I^{131} from the thyroid glands of untreated and propylthiouracil-treated rats. The "zero-time" count was made 40 hours after the injection of I^{131}.

FIG. 1B. The release of I^{131} from the thyroids of untreated rats. The "zero-time" count was made *30* hours after the injection of I^{131}.

TABLE 1. LOSS OF I^{131} FROM THE THYROIDS OF NORMAL AND PROPYLTHIOURACIL-TREATED RATS

Interval after zero time*	Normal rats		Propylthiouracil-treated rats	
	No. of rats	Per cent of zero time* value and standard error	No. of rats	Per cent of zero time* value and standard error
10	6	97.9†	6	93.0†
24	17	81.3±1.48	20	69.8±2.97
48	17	65.7±0.96	20	42.2±2.13
72	17	52.6±1.49	20	27.9±2.12
96	10	42.2±1.97	14	17.6±1.73
120	10	36.2±1.90	14	10.3±1.38
144	4	34.0†		
168	3	26.6†		

* The zero time determination was carried out 40 hours after the injection of I^{131}.
† The standard error was calculated only when ten or more determinations were made.

evident that the loss of I^{131} from the thyroid was considerably more rapid in the propylthiouracil-treated rats than in the untreated animals. The biological half-lives, here determined graphically from the intercepts on the 50 per cent line are 3.3 days and 1.6 days respectively for the normal and propylthiouracil-treated rats.[5] Factors which could be responsible for this two-fold difference in release of I^{131} from the thyroid gland are:

(1) Dilution by newly accumulated I^{127}. For example, at zero time the specific activity is of a certain magnitude and delivery of a constant amount of hormone to the circulation would mean loss of a constant amount of I^{131} if the specific activity remains the same. Since newly synthesized thyroxine would, however, reduce the specific activity, a smaller loss of I^{131} would occur despite maintenance of the same rate of delivery of hormone. That is, the difference might be due to the block in the synthesis of *new* organic iodine caused by propylthiouracil.

(2) Reduction of the plasma protein-bound iodine in the propylthiouracil treated rats with a consequent hypophyseal activation of the thyroid and hence an increased rate of release of I^{131}. It must be pointed out that the thyroid weights of these animals at death were 30 per cent greater than the normal thyroid weights.

In one experiment the zero time determination was made 30 hours after the I^{131} injection instead of 40 hours. At this time significant I^{131} accumulation in the thyroid was still occurring and the release curve (Figure 1B) showed first a slight increase and then a lag of about ten hours behind curves for which zero time occurred at 40 hours after the I^{131} injection. When, therefore, the ordinate was transposed by ten hours, the resulting parallel line went through the origin (zero time and 100 per cent) and the biological half-life was again 3.4 days. The increase resulting in these untreated animals could, furthermore, be abolished by dilution of the residual radioactive iodine in the circulation by the injection of small amounts of potassium iodide (10 μg. I^- every eight hours). Under these conditions the initial rise above 100 per cent failed to occur and the biological half-life was 3.2 days.

2. *The Effect of Hypophysectomy.* Twelve Sprague-Dawley male rats were injected intraperitoneally with 30 μc. of I^{131} as iodide. Forty hours thereafter zero time determinations were carried out and each rat received 10 mg. of a propylthiouracil suspension intraperitoneally. The rats were then divided into two groups. The rats of the first group were hypophysectomized by the parapharyngeal route[6] and were then fed the 0.03 per cent

[5] The correction for isotopic decay has already been made, and the biological half-life as used here is thus independent of the decay properties of the isotope.

[6] We are grateful to Dr. Richard W. Payne of this department for performing these operations.

propylthiouracil diet. The rats of the other group were sham-operated and then received the same diet. The results are depicted in Figure 2. The biological half-life of I^{131} in the thyroids of hypophysectomized rats fed propylthiouracil was about 24 days whereas the half-life of the I^{131} in the thyroids of the control animals was 1.7 days, the latter value being in good agreement with the over-all average of 1.6 days.

After five days the hypophysectomized rats received 10 Junkmann-Schoeller units of thyrotropic hormone[7] by subcutaneous injection. In

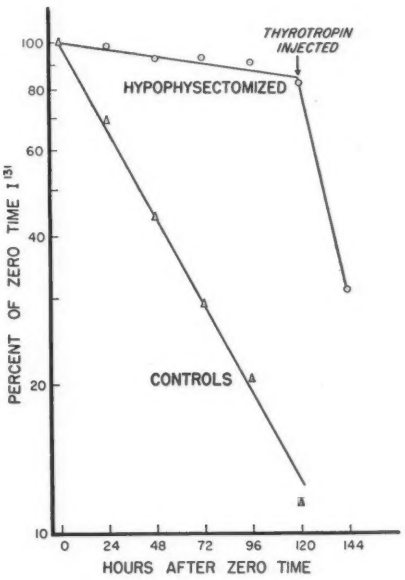

FIG. 2. The effect of hypophysectomy on the release of I^{131} from the thyroids of propylthiouracil-treated rats. The "zero-time" count was made 40 hours after the injection of I^{131}. Thyrotropin was administered in a single dose on the fifth day.

24 hours three to four times as much I^{131} was lost from the glands as had been lost during the preceding five-day period. The slope during this interval exceeded that of the controls and approximated that of the thyrotropin-treated normal animals to be presented in a later section. It is felt that this is a direct demonstration of the intimate role played by thyrotropin in the release of iodine from the thyroid gland.

3. *The Effect of Thyroxine Administration.* Ten Sprague-Dawley rats were injected intraperitoneally with 30 μc. of I^{131} and following a zero time count 40 hours later, all received an intraperitoneal injection of 10 mg. of propylthiouracil in saline suspension. They were then divided into two

[7] The thyrotropin was supplied through the kindness of Dr. D. McGinty of Parke, Davis and Company, Detroit, Michigan.

groups; the first received 15 μg. of *dl*-thyroxine (dissolved in a minimum of NaOH) by the intraperitoneal route; the second group served as controls—they received an equivalent amount of iodide as potassium iodide.

From the results plotted in Figure 3 it is apparent that under the influence of thyroxine a marked depression occurred in the release of I^{131}

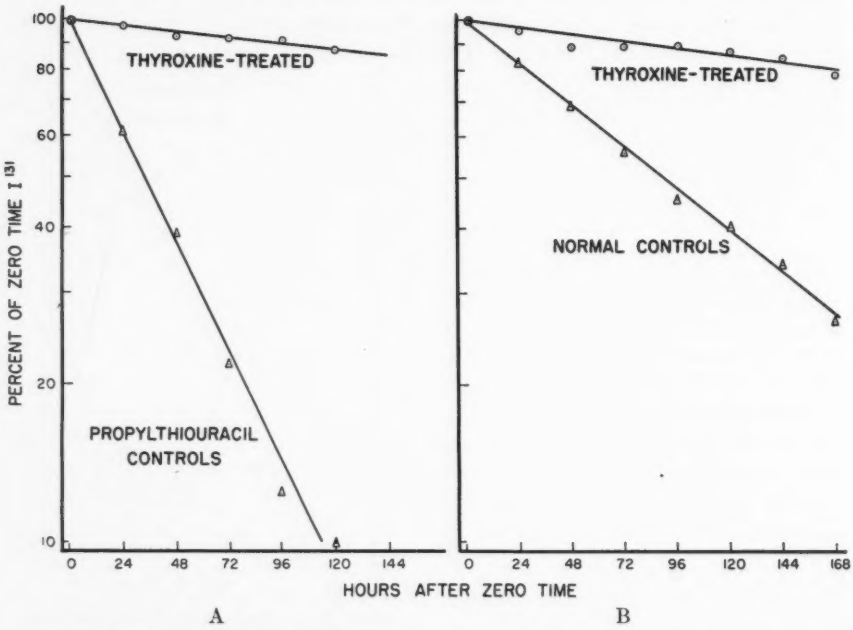

FIG. 3A. The effect of thyroxine on the release of I^{131} from the thyroids of propylthiouracil-treated rats. The controls received equivalent amounts of iodide. The "zero-time" count was made 40 hours after the administration of I^{131}.

FIG. 3B. The effect of thyroxine in the absence of propylthiouracil. The controls received equivalent amounts of iodide. The "zero-time" count was made 40 hours after the injection of I^{131}.

from the gland. As early as 24 hours thyroxine was able to inhibit the release of I^{131} from the thyroid, limiting this loss to about 3 per cent of the original I^{131} whereas the control figure was nearly 40 per cent.

The biological half-life of the thyroid I^{131} in the control animals, receiving a single daily injection of 10 μg. of iodide, was 1.4 days while that of the thyroxine-injected rats was about 26 days. This finding was not unexpected in view of the 24 day half-life reported above for the hypophysectomized rat.

A second experiment was carried out on ten Sprague-Dawley male rats. This experiment was identical in all respects to the above experiments except that synthesis was not blocked by the administration of propylthiouracil. The results were essentially similar (Figure 3B), the biological half-lives being 3.7 and 24 days respectively. Since the interpretation of these results is complicated by such factors as mentioned in part 1, the graph is given here merely for comparison.

4. *The Effect of Thyrotropic Hormone.* At least two acceleratory functions have been ascribed to thyrotropic hormone in its action on the thyroid gland: an increase in the iodine-concentrating capacity and an increased rate of release of hormone from the gland. Administration of thyrotropin

TABLE 2. THE EFFECT OF THYROTROPIC HORMONE ON THE RELEASE OF I^{131} FROM THE THYROID GLAND

Interval after thyrotropic injection and zero time*	Mean Per cent of thyroid I^{131} remaining after zero time	Standard error of the mean*
hours	per cent	
1	98.5	0.97
2	95.1	1.49
3	88.9	2.16
4	77.7	1.53
7	56.2	2.10

* The zero time determination was carried out 40 hours after injection of the I^{131}.
† The standard error was calculated only when ten or more determinations were made.

will lead to marked reductions in thyroidal iodine and VanderLaan and Greer (1950) have recently shown in hypophysectomized rats that this effect is more rapid when iodine accumulation, which is also stimulated by thyrotropin, is blocked by propylthiouracil administration. Since Stanley and Astwood (1949) had shown that a "latent period" of about eight hours had to elapse before any effect of thyrotropin could be noticed on iodine *accumulation* in human subjects, it became of interest to determine the rapidity with which this hormone exerts its effect on *release* of iodine from the gland in the rat.

Seventeen male Sprague-Dawley rats were injected with 35 μc. of I^{131} and the initial count was carried out 40 hours later. They then received 10 mg. of a propylthiouracil suspension and one group was set aside as a control group. The other rats received 10 units of thyrotropin subcutaneously. They were then counted externally at frequent intervals. The actual values of thyroid I^{131} are listed with standard errors in Table 2.

Since it was difficult to define a minimum significant loss of I^{131} and since the standard errors ranged from about one to two (1–2 per cent), a 10

per cent loss in thyroid I^{131} was chosen as the minimum significant change brought about by thyrotropic hormone, that is, $p < 0.01$ at 2 hours $p < 0.02$ and at 3 hours $p < 0.01$.

It can readily be discerned from Table 2 and Figure 4 that the first significant effect of thyrotropin (as defined here) occurred sometime between two and three hours after the injection of this hormone. While it might be argued that this is a continuous process starting at zero time, the shape of the curve in Figure 4 does not support such an assumption. Furthermore, the eight-hour latent period found by Stanley and Astwood for the thyrotropic effect on iodine accumulation in the human subject supports the view that here too there is a delay or latent period in the action of thyrotropin.

5. *The Effect of Iodide.* It has recently been demonstrated in several laboratories that iodide, when administered to rats (Wolff and Chaikoff

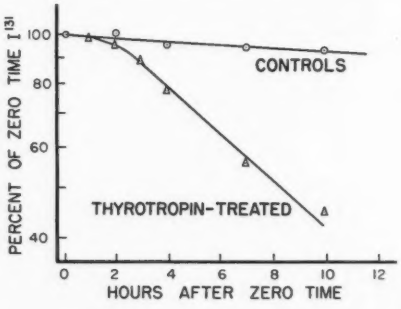

FIG. 4. The effect of thyrotropin on the release of I^{131} from the thyroids of propylthiouracil-treated rats. The "zero-time" count was made 40 hours after the injection of I^{131}.

1948a, b, c; Raben, 1949) or human subjects (Stanley, 1949) in pharmacological quantities, effectively inhibits the formation of organic iodine in the thyroid gland. While this finding offered promising evidence for an explanation for the therapeutic effect of iodine in Graves' disease, it was soon found that this inhibition could not be prolonged in rats beyond about two days and that thereafter organic iodine began to accumulate despite the maintenance of high plasma concentrations of iodide (Wolff *et al.*, 1949). Additional mechanisms have therefore been sought to explain this paradoxical action of large doses of iodide. The present method offered an opportunity to test release of I^{131} as influenced by iodide administration and the results of such a study are reported below.

Since high concentrations of iodide can block the synthesis of thyroidal organic iodine for a limited period, no propylthiouracil was given in the initial experiments of this section. Forty-five Sprague-Dawley males received 35 μc. of I^{131} intraperitoneally and 40 hours later they were divided into the following groups:

1. Zero time controls (untreated) 7
2. Controls (untreated)—three groups of six killed two, three, and four days thereafter 18
3. Iodide group—receiving 750 μg. of iodide as potassium iodide every eight hours after an initial injection of 1,000 μg. 18

The rats were killed by exsanguination under sodium pentobarbital anes-

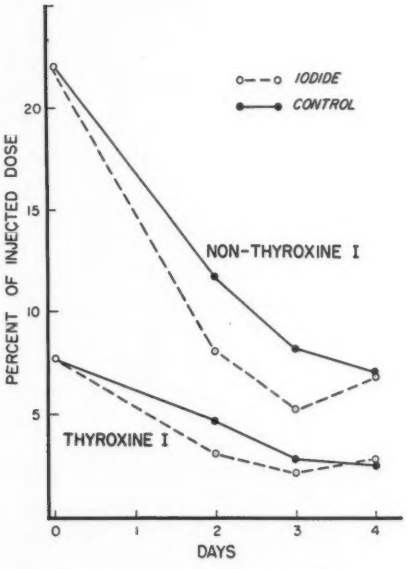

FIG. 5. Decrease of thyroidal thyroxine and non-thyroxine organic-I^{131} under the influence of large doses of iodide (750 micrograms three times daily).

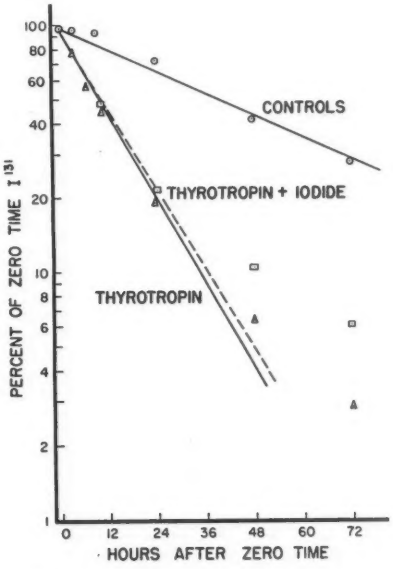

FIG. 6. The effect of thyrotropin on the release of I^{131} from the thyroids of propylthiouracil-treated rats when given with and without simultaneous iodide treatment (500 micrograms three times daily). The "zero-time" count was made 40 hours after the injection of I^{131}.

thesia and the thyroids were rapidly removed and weighed. The separation of iodine into organic and inorganic fractions, and the subsequent fractionation of the organic fraction, have been described in earlier reports (Taurog and Chaikoff, 1946; Wolff and Chaikoff, 1948a).

The results are plotted in Figure 5. It is clear that the plasma iodide, which is known to be high under these conditions (Wolff *et al.*, 1949) failed to influence in any marked manner the thyroxine or non-thyroxine organic I^{131} contents of the thyroid glands.

Several identical experiments were carried out all with similarly negative

results. Previous activation of the thyroid glands by propylthiouracil feeding with subsequent cessation of treatment, as well as propylthiouracil treatment after the zero time determination had been made, did not reveal any depression in the release of I^{131} by excess iodide.

Since numerous reports in the literature have claimed (Friedgood, 1935; Anderson and Evans, 1937; VanderLaan *et al.*, 1941) that the administration of large doses of iodide can abolish or interfere with the effects of administered thyrotropin, further studies on the iodide effect were carried out in conjunction with thyrotropin treatment. The experimental procedures were the same as above except that 10 units of thyrotropin were injected at eight-hour intervals. When iodide was given, 500 μg. was administered at the same time as the thyrotropin but by the intraperitoneal rather than by the subcutaneous route.

TABLE 3. THE EFFECT OF IODINE AND THYROTROPIC HORMONE ON THE RELEASE OF I^{131} FROM THE THYROID GLAND

Interval after zero time determination	Per cent of zero time I^{131} remaining in thyroid gland	
	Thyrotropin & 10 μg. I^-	Thyrotropin & 500 μg. I^-
hours	per cent	per cent
4	79.3†	78.3
18	39.4	39.5
48	20.5	20.3
68	11.7	15.4
92	8.3	11.5

† Each point is the average of five determinations on as many rats.

The results shown in Figure 6 demonstrate again the rapid loss of I^{131} from the thyroid following injections of thyrotropin. The biological half-life, which for the controls of this experiment was 1.7 days, was shortened to eight to ten hours (see also Figure 4). The deviation from a straight logarithmic curve at the 48 and 72-hour intervals remains unexplained.[8]

When 500 μg. of iodide was given three times daily with the thyrotropin, the drop in I^{131} from the thyroids of the rats of this group occurred at the same rate as in the absence of iodide (*although at 72 hours* $p<0.1$ *and* $p>0.05$). The biological half-life in this case was also about ten hours. A similar unexplained deviation from the straight line relationship occurred.

Since the possibility existed that propylthiouracil might in some way interfere with the action of iodine (Albert *et al.*, 1947), another experiment was carried out where this antithyroid drug was not used. Of two groups

[8] The resemblance of this deviation to a two-component curve lends credence to the possibility that the propylthiouracil block was not complete under these conditions of extreme stimulation with a resulting retardation of I^{131} loss either by dilution with newly accumulated I^{127} or from reaccumulation of I^{131}.

of five rats, one received 10 units of thyrotropin and 10 μg. of iodide three times daily (by different routes). The second group received 10 units of thyrotropin and 500 μg. of iodide. The points show good agreement and their averages are therefore listed in table form (Table 3). The biological half-life in each case was about thirteen to fourteen hours. The increase in half-life values over propylthiouracil animals was expected in view of the results cited in Section 1. Again it was evident that large doses of iodide were not able to abolish the effect of simultaneously administered thyrotropin under the conditions of these experiments.

Histologically however, iodide reduced the hypertrophy induced by thyrotropin. With only a single exception thyroids of the various groups could be arranged in decreasing order of hypertrophy: thyrotropin only, thyrotropin plus iodide, control.

DISCUSSION

In the present study it was not possible to measure loss of thyroid hormone *per se*. However, from studies of the blood iodine (Taurog *et al.*, 1948, 1950; Rosenberg, 1950), it is likely that most, if not all, of the organic iodine that is delivered to the circulation is in a form closely related to thyroxine. The decrease in I^{131} in the thyroid may therefore be considered to reflect secretion of I^{131}-labeled thyroxine. The conversion of monoiodotyrosine or diiodotyrosine I^{131} to thyroxine I^{131} would not influence the results obtained by external counting. One factor that could be of importance is the loss of inorganic I^{131} from the gland. This is known to occur as a result of exchange with iodide (I^{127}) or from the administration of large doses of thiocyanate. It must be pointed out, however, that 40 hours after the injection of a tracer dose of radioactive iodine, over 95 per cent of the thyroid I^{131} is in organic (non-exchangeable) combination. Thus, such factors are of relatively minor importance here, even in the cases where large doses of iodide (I^{127}) were given.

Estimates of the (chemical) amounts of thyroxine delivered to the circulation can be made from information revealed by specific activity-time data. Variations in I^{127} content that exist from gland to gland are too extensive to permit calculations of thyroxine specific activities from values of thyroxine iodine in the literature. Nevertheless, such an attempt has recently been made (Wolterink and Lee, 1950).

Furthermore, according to the criteria set forth by Zilversmit *et al.* (1943), the existence of a steady state is one of the prerequisites for this type of turnover calculation from specific activity. In the present study, however, renewal has been blocked by propylthiouracil or iodide; thus the conditions that prevail are more like those of excretion of a fixed amount of material. The results presented here are indicative only of loss of

accumulated I^{131} from the gland and quantitative chemical (I^{127}) considerations can only be inferred.

SUMMARY

Variations in the release of I^{131} from the thyroid gland have been determined by means of external gamma counting in rats subjected to a number of experimental conditions. In most cases I^{131} was injected forty hours before the first count was made and propylthiouracil treatment was then instituted to block further synthesis of organic iodine. Under these conditions release of thyroidal I^{131} constituted a more meaningful measurement.

Control (untreated) rats lost I^{131} from the thyroid with a biological half-life of 3.3 days. Addition of 0.03 per cent propylthiouracil to the diet accelerated release to a half-life of 1.6 days.

Hypophysectomy resulted in a prolongation of the half-life to about 24 days. Subsequent treatment of these animals with thyrotropin led to a rapid recovery of the release to normal or greater than normal values in 24 hours.

Treatment with 15 micrograms of thyroxine daily had an effect similar to hypophysectomy. In the presence of propylthiouracil, thyroxine prolonged the biological half-life of I^{131} in the thyroid to 26 days; controls receiving an equivalent amount of iodide had a 1.4 day half-life. In the absence of propylthiouracil the thyroxine and control values were 24 and 3.7 days respectively.

Thyrotropin treatment markedly accelerated the release of I^{131} from the thyroid. Whereas propylthiouracil controls lost I^{131} from the thyroid with a half-life of about 1.7 days, the thyrotropin-treated rats did so with a biological half-life of only eight to ten hours. In addition, it was demonstrated that the action of thyrotropin showed a latent period of two to three hours before any significant effect could be shown on release of I^{131} from the thyroid.

Large doses of iodide failed to influence the release of previously injected I^{131} from the gland. This could be shown both in the thyroxine and non-thyroxine organic iodine fractions of the thyroid. Furthermore, iodide was unable to alter significantly the accelerated release of I^{131} brought about by simultaneously administered thyrotropin.

Some limitations of this type of measurement are discussed.

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PREVENTION AND REVERSAL DESPITE HYPERGLYCEMIA OF GLYCOGEN INFILTRATION ("HYDROPIC DEGENERATION") IN THE PANCREAS IN ALLOXAN DIABETES IN THE RABBIT[1]

G. LYMAN DUFF AND W. E. TORESON

From the Department of Pathology, Pathological Institute, McGill University, Montreal, Canada

IN so-called "hydropic degeneration" of the pancreatic islets and ductular epithelium in experimental and human diabetes mellitus, glycogen can be demonstrated readily in the vacuolated cells by common histological techniques (Toreson, 1950). The characteristic vacuolar appearance of swollen pancreatic cells affected by "hydropic degeneration" is attributable to artefactual removal of intracytoplasmic accumulations of glycogen rather than of excessive quantities of water (Weichselbaum and Stangl, 1902; Allen, 1913; Homans, 1915; and others) or of serous fluid (Ogilvie, 1949). This pancreatic lesion would be designated more precisely and distinctively by the term *glycogen infiltration* and we propose to use the term because we believe that careful re-examination of the various materials of previous authors would show the presence of glycogen in "hydropic" pancreatic cells in each instance.

Glycogen infiltration of the islets of Langerhans is the sole histopathological feature common to all forms of permanent experimental diabetes with the exception, of course, of that due to total pancreatectomy (Allen, 1913; Homans, 1914, 1915; Richardson and Young, 1938; Richardson, 1939–1940; Ham and Haist, 1941; Lukens and Dohan, 1940, 1941a, b, 1942; Goldner and Gomori, 1943; Duff, McMillan and Wilson, 1947; Kadota, 1950; Kobernick and More, 1950) and of human diabetes mellitus (Weichselbaum and Stangl, 1901, 1902; Weichselbaum, 1910; Kraus, 1929; Warren, 1938). The affected cells persist for varying periods of time in different species. The development of the lesion may be prevented by various means and restoration of the structural integrity and sometimes the functional capacity of the affected cells is possible (Allen, 1913, 1922a, b; Copp and Barclay, 1923; Haist, Campbell and Best, 1940; Campbell,

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Haist, Ham and Best, 1940; Lukens and Dohan, 1940, 1941b, 1942; Lukens, Dohan and Wolcott, 1943; Duff, McMillan and Wilson, 1947). Allen (1913, 1922b) believed that a deficient amount of circulating hormone (i.e. hypoinsulinemia) might provide the stimulus to functional overstrain, which he originally conceived to be the factor responsible for structural deterioration of the affected islet cells. On the other hand, Lukens and associates (Lukens and Dohan, 1941a, b, 1942; Lukens, Dohan and Wolcott, 1943; Lukens, 1946; Dohan and Lukens, 1947, 1948) have repeatedly attributed to hyperglycemia *per se* a preponderant role in the pathogenesis of "hydropic degeneration," but recently they (Lukens, 1946; Dohan and Lukens, 1948) have modified this point of view to admit the possible importance of other unknown factors.

If glycogen infiltration in the pancreatic islets of the diabetic animal is a consequence of prolonged, excessive, functional strain referable to hypoinsulinemia, provision of exogenous insulin in small amounts inadequate to lower blood sugar levels appreciably might prevent development of the lesion or allow restoration of the affected cells to normal appearance. The studies reported here were designed to attempt confirmation of the previous observations of "hydropic change" in the pancreas of alloxan diabetic rabbits reported by Duff, McMillan and Wilson (1947) and to determine whether beneficial effects on these pancreatic lesions could be produced by small doses of insulin.

MATERIALS AND METHODS

Adult, white, domestic rabbits, purchased locally, were housed in separate metabolism cages. During a period of two weeks observations of general health and two fasting blood sugar determinations were made on each animal. Constant access to Purina Rabbit Chow and fresh water was unrestricted throughout the course of the experiments. After preliminary observations had established the apparent normality of the health of the rabbits, and when their body weights were between 1.80 and 3.50 kilograms they were given intravenous injections of alloxan (alloxan monohydrate, Eastman Kodak Co.). A freshly prepared, non-sterile solution of 5 grams of alloxan in 100 mls. of distilled water was used. Each rabbit received the full dose via the marginal vein of the pinna within 30 seconds to 2 minutes. The usual dose was 200 mg. of alloxan per kilogram of body weight, but a few animals were given 175 mg./kilo (T53 and T55), and several were given 150 mg./kilo (T64, T67, C89, and C91). Hypoglycemic convulsions were avoided by administering 25 grams of dextrose in 50 mls. of water by stomach tube about six to eight hours after the injections of alloxan.

During periods of 8 to 48 weeks following the injection of alloxan the diabetic state of each rabbit was studied by frequent determinations of blood and urine sugar content, ketonuria, and body weight. Eleven pancreatic biopsies were studied after periods of untreated alloxan diabetes varying from 12 to 26 weeks. After biopsy ten rabbits were treated with insulin in varying doses over varying periods of time. During treatment frequent or daily blood sugar determinations were made on "random" samples collected immediately before giving the insulin. The twenty-four hour urine volume was measured

daily and its glucose content was determined. At the end of the course of treatment a second pancreatic biopsy was studied or autopsy was performed.

Five rabbits were given a small dose of insulin once each day beginning on the second day after injecting alloxan and continuing for 5 to 16 weeks. Daily observations of random blood sugar level, glucosuria, ketonuria and body weight were made during the first 5 to 9 weeks. Two of these animals were studied at autopsy after 5 and 6 weeks. The pancreases of the others were examined at biopsy after 12, 15, and 16 weeks. For the latter group, the daily dose of insulin was then altered for an additional period of 3 weeks, after which complete autopsy examinations were made.

Fasting blood sugar samples were taken after the rabbits had been without food (Purina Rabbit Chow) for 16 hours. "Random" samples were always taken while the rabbits had free access to food; during therapy these samples were collected immediately before giving the daily injections of insulin. All of the blood samples were taken from the central artery of the pinna. Except when fasting analyses were to be made, all of the rabbits had unlimited supplies of food and water; the amounts consumed were not recorded. Urine samples were collected from funnel-type, screen-floored cages in one-litre glass bottles. Twenty-four hour specimens were measured at approximately the same hour each morning.

Blood sugar levels were determined by Nelson's (1944) photometric adaptation of the Somogyi method. Qualitative estimates of glucosuria and ketonuria were made with "Galatest" and "Acetone Test" (the Denver Chemical Co., Montreal). Quantitative analyses of urine glucose content were made by Benedict's titration method on portions of twenty-four hour specimens.

All of the rabbits that were treated received protamine zinc insulin in a single dose given each morning. One rabbit (T4) received both morning and evening injections and required supplementary doses of crystalline zinc insulin to attain adequate control of blood sugar level and urine glucose excretion.

Histological studies were made on Helly-fixed, paraffin-embedded tissues sectioned at from 3 to 6 microns and stained with hematin, phloxin and saffron. Pancreatic sections were also stained by the Gomori method for islet cell granules and by several methods for demonstrating glycogen. The latter included both the Hotchkiss (1948) and the McManus (1948) versions of the periodic acid-Schiff's reagent reaction, Bauer's chromic acid-Schiff's reagent technic as described by Lillie (1948) and the traditional carmine method of Best. It was found unnecessary to use alcoholic fixatives or celloidin films in these experimental studies (Toreson, 1950). Although complete gross and microscopic autopsy examinations were made, observations pertinent to the present studies are limited to the histopathology of the pancreas.

OBSERVATIONS

Seventeen diabetic rabbits were studied for periods of 8 to 48 weeks after injection of alloxan. Insulin treatment was withheld during this time. One rabbit, number T4, received sufficient insulin to control hyperglycemia and glucosuria during the 13th to 17th weeks, inclusive; upon discontinuing insulin injections further observations were made during 16 weeks of relapse. Seven of these untreated, alloxan diabetic rabbits showed glycogen infiltration in the pancreas at autopsy when found dead or killed. Eleven biopsy specimens from 10 untreated rabbits (2 from num-

ber T4) showed the same lesion. Metabolic data for all of the animals have been summarized in Table 1. The amounts of gain or loss of body weight, the average values of fasting and random blood sugar levels, polyuria and glucosuria were not apparently related to the duration of diabetic symptoms. About half of the diabetic rabbits gained weight and half lost weight; the range of loss was similar to the range of gain and the average loss was

TABLE 1. DIABETIC CHARACTERISTICS OF RABBITS SURVIVING INJECTION OF ALLOXAN FROM 8 TO 48 WEEKS

Rabbit No.	Duration of Diabetes	Change in Body weight (Kilograms)		Average blood sugar level (mg. %)		Average Urine Excretion	
	Weeks	Gain	Loss	Fasting	Random	ccs./day Volume	Gms./day Glucose
T53-A	8		0.92	365	557		
T4-B1	12	0.35		414			
T6-B1	12		1.22	442			
T37-A	13	1.00		463	549		
T64-B1	13	0.46		440	547	700	31.1
T67-B1	13	0.40		354	458	528	28.4
T15-A	15		0.31	376		875	24.7
T55-A	15		0.47	458	533	616	25.6
T4-B3	16*		0.39	555	469	490	15.2
T16-B1	17	0.67		470		950	25.7
T17-B1	17		0.50	463	645	1000	15.0
T40-A	17		0.15	495	576	458	21.1
T25-B1	20	0.31		518		460	16.6
T20-B1	21	0.61		546		800	25.6
T21-B1	22	0.73		420		850	
T23-B1	26	0.39		485		525	15.0
D75-A	28	0.03		246	390	601	21.5
B98-A	48		0.42	595	579	878	37.6
Mean		0.50	0.55	450	530	695	23.4
Min.	8	0.03	0.15	246	390	458	15.0
Max.	48	1.00	1.22	595	645	1000	37.6

* Values represent 18th to 34th weeks, inclusive (i.e. following discontinuation of insulin treatment given from 13th to 17th weeks, inclusive).

almost identical with the average gain. The average values of the fasting and random blood sugar levels of the rabbits that gained weight were not appreciably different from the values of the rabbits that lost weight. Similarly, glucosuria was not more massive in the group that lost than in the group that gained weight, nor was its severity apparently conditioned by the degree of elevation of the blood sugar level. However, it may be noted that rabbit number B98, which was studied for the longest period, did show the highest average fasting blood sugar level and the greatest average glucosuria. This rabbit was unusual also in that if manifested continuous persistence of ketonuria throughout the study. In all of the biopsies and autopsies pancreatic glycogen infiltration (so-called "hydropic degenera-

tion") was present (Fig. 1). Islets and ductules were always affected in each instance, although sometimes with apparently different degrees and extents. Nine pancreases, including six biopsy fragments, showed cytoplasmic accumulations of glycogen in cells situated in centro-acinar positions. Mitotic figures were seen in ductular epithelial cells in three biopsy specimens; in one of these pancreases mitoses were also present in centro-acinar cells, acinar exocrine cells and agranular (apparently beta type) islet cells. Lobules of pancreatic tissue having an hyperplastic appearance, resembling the embryonic structure, were encountered in each of the three samples which had apparent mitotic activity and in one other biopsy. Glycogen could be demonstrated with facility in the renal tubular epithelium in all of the autopsy specimens.

At the end of the periods of untreated diabetes, ten rabbits received insulin therapy. In three of these experiments (rabbits numbers T21, T6 and T16), only 4, 10 and 32 units of insulin were given during one or two days subsequent to pancreatic biopsy. None of these rabbits showed appreciable differences in extent or degree of glycogen infiltration in the pancreas at autopsy as compared with biopsy.

In three experiments good control of hyperglycemia and glucosuria was achieved and maintained by administering sufficient insulin. Duff, McMillan and Wilson (1947) previously attained similar results in one rabbit. On that occasion during the first 20 days of insulin treatment daily deter-

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PLATE I. Figures 1, 2, 4, 6, 7.

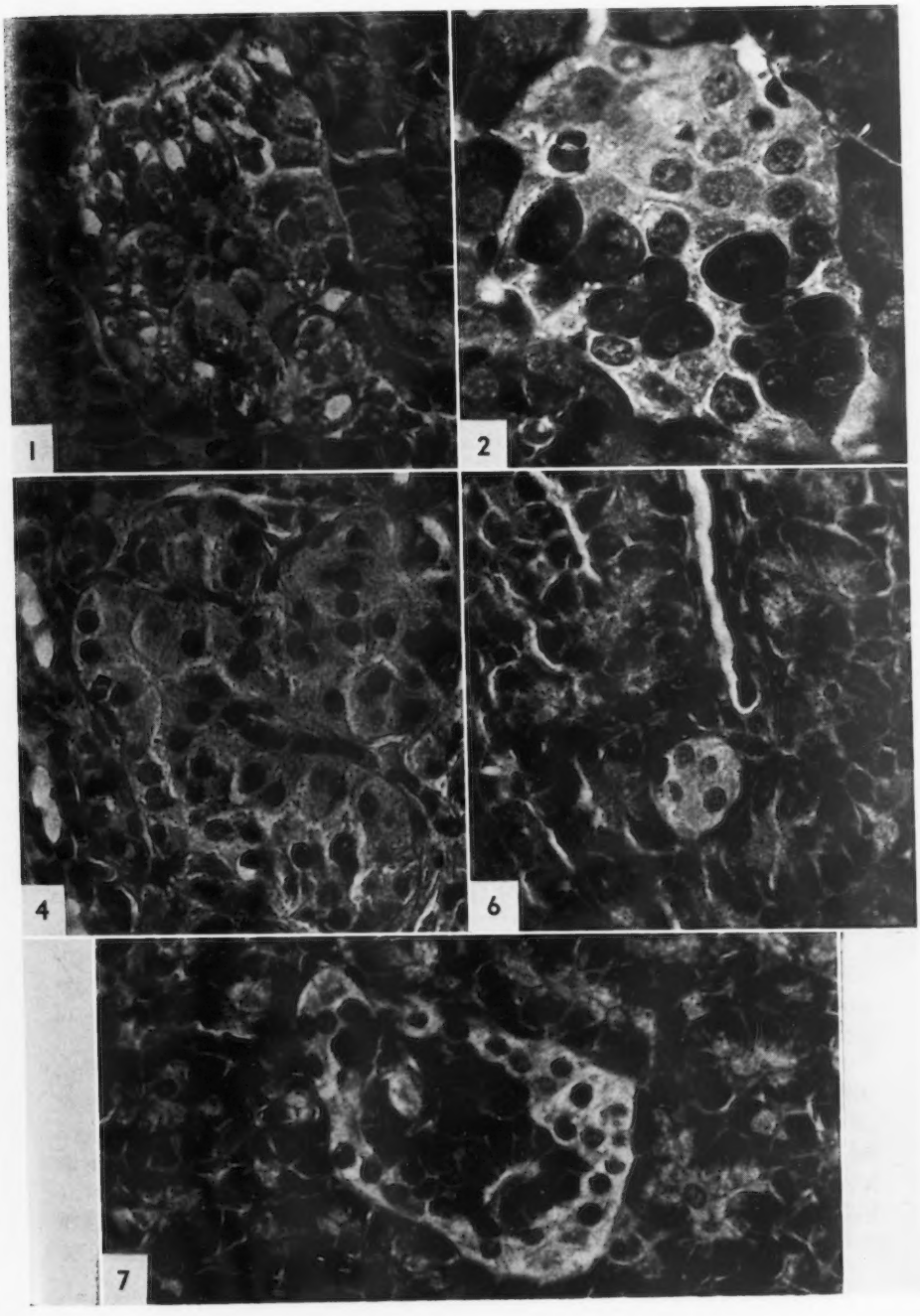
FIG. 1. Glycogen infiltration of an islet of Langerhans from alloxan diabetic rabbit number D75. Autopsy section prepared 6 months after a single intravenous dose of alloxan (200 mg. per kilo of body weight). Average fasting blood sugar level 246 mg.%; average random blood sugar level 390 mg.% The glycogen appears as heavy black linear or crescentric deposits near the cell membranes. Best's carmine. Helly's fluid; ×530.

FIG. 2. Restoration of islet cells previously affected by glycogen infiltration to normal appearance by treatment with large doses of insulin. The large dark cells are alpha cells. The others are beta cells which have full complements of cytoplasmic granules. Zenker-formol fixation; Gomori's stain; oil immersion, ×1,100.

FIG. 4. Restoration of islet cells previously affected by glycogen infiltration to normal appearance by treatment with such small doses of insulin that hyperglycemia was present continuously during therapy (see Fig. 3). Rabbit number T67, autopsy section. Helly's fixation; Gomori stain; ×500.

FIG. 6. Prevention of development of glycogen infiltration in a small islet and ductule of an alloxan diabetic rabbit, number C89, given such small doses of insulin that hyperglycemia was continuously present over a period of 4 months (see Fig. 5). Helly's fixation; Gomori stain; ×530.

FIG. 7. Rapid development of glycogen infiltration in an islet of an alloxan diabetic rabbit, number C49, within 3 weeks after discontinuation of treatment with small doses of insulin. Helly's fixation; Gomori stain; ×530.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minations of the blood sugar level showed that hyperglycemia was present almost continuously (average value 390 mg. per cent). Qualitative urinalyses during the ensuing, final 10 days of insulin therapy disclosed only occasional traces of glucose. The pancreatic islets and ductules had shown obvious "hydropic degeneration" at biopsy before insulin treatment; afterwards the cells were found to have normal structural appearances. In autopsy sections of the pancreas many islets contained numerous agranular cells together with others in which some beta granules were present. Some islets contained cells that were indistinguishable from normal beta cells. In the present studies rabbit number T17 experienced hypoglycemic convulsions in response to insulin injections on three separate occasions during a two-week course of therapy. Only the slightest persistence of glucosuria was found. Although extreme glycogen infiltration was noted in the pre-treatment biopsy of the pancreas, only a very few glycogen-filled ductular epithelial cells could be demonstrated at autopsy and neither islet cells nor centroacinar cells were found affected. Rabbit number T4 responded to insulin therapy by attaining and maintaining normal blood sugar levels throughout most of 27 days. The obvious lesions present in the islets and ductules at pre-treatment biopsy had disappeared completely in a post-treatment pancreatic biopsy. When insulin therapy was discontinued, random blood sugar levels rose only gradually to more than 500 mg.%; fasting levels during the later weeks of relapse had higher values than the random levels determined earlier. In a third pancreatic biopsy taken from this same rabbit after 16 weeks of relapse, extreme and widespread glycogen infiltration was present together with appearances suggesting proliferative regeneration. A second course of insulin treatment was given for 59 days; during this period the average value of the blood sugar level was 217 mg.% and there was frequently more than one gram of glucose per 100 mls. of urine. Nevertheless, at autopsy no glycogen could be found in the pancreas. Also, the appearance of proliferative regeneration was not evident.

Glycogen nephrosis was not apparent in the kidneys of any of these insulin-controlled diabetic rabbits at autopsy. In each instance the restored pancreatic islets of Langerhans contained numerous examples of granular beta type cells (Fig. 2).

Four diabetic rabbits, numbers T23, T20, T64 and T67, received insufficient insulin to control hyperglycemia and glucosuria during periods of 10 to 49 days. The metabolic and histopathologic data for these experiments are presented in Table 2. Despite average random blood sugar levels greater than 321 mg.% during the period of treatment the pancreatic lesions were distinctly less severe and less extensive after treatment than in biopsies taken before treatment. Rabbits numbers T23, T64, and T67

showed persistence of mere traces of glycogen in the cytoplasm of a few islet cells but some of the ductular epithelial cells of the latter two contained conspicuous accumulations of glycogen. The pancreas of number T20 contained at autopsy no demonstrable glycogen despite an average random blood sugar level of 415 mg.% during the treatment period. The metabolic

TABLE 2. EFFECTS OF INSULIN ON BLOOD SUGAR, GLUCOSURIA, WEIGHT AND PANCREATIC GLYCOGEN INFILTRATION IN ALLOXAN DIABETIC RABBITS: RESTORATION DESPITE HYPERGLYCEMIA

Rabbit number	T23	T20	T64	T67-B_1B_2	T67-B_1A*
Duration of diabetes (weeks)	26	21	13	13	
Total insulin (units)	180	1722	140	114	210 (96)
Duration of treatment (days)	10	36	20	29	49 (20)
Average Glycemia (mg. %)					
before R_1 fasting	485	546	440	354	354
before R_1 random	—	—	547	458	458
during R_1 random	367	415	489	374	355 (321)
Average Glucosuria (gms./day)					
before R_1	15.0	25.6	31.1	28.4	28.4
during R_1	6.8	7.4	13.2	7.1	— (—)
Change in Weight during R_1 (kilograms)	−0.08	+0.59	+0.29	−0.03	−0.01 (+0.02)
Glycogen Infiltration					
before R_1					
islets	some	extreme	some	some	some
ductules	some	extreme	some	some	some
centro-acinar	none	some	none	none	none
after R_1					
islets	trace	none	trace	trace	none
ductules	none	none	some	some	none
centro-acinar	none	none	none	none	none

* T67-B_1A: In this column the average values for the entire treatment period are given together with the figures representing the 20 additional days of therapy (in parentheses) for comparison with the first 29 days as given in column T67-B_1B_2.

course of rabbit number T67 is depicted graphically (Fig. 3). Although daily injection of 4 units of protamine zinc insulin during a period of 29 days failed to restore completely the structural integrity of the affected pancreatic cells, continuation of very small doses of insulin for an additional 20 days resulted in the complete disappearance of glycogen from the pancreas. Some islet cells were found to have complete complements of beta cytoplasmic granules (Fig. 4), although most of them were of alpha and agranular types. In this experiment the average value of daily random

blood sugar level for the entire treatment period (49 days) was 355 mg.%; during the last 20 days it was 321 mg.%.

Five rabbits made diabetic by single intravenous injections of alloxan were given small doses of insulin daily for periods of 5 to 16 weeks beginning immediately after the secondary elevation of blood sugar level was manifest. All of these animals showed sustained, severe hyperglycemia of more than 354 mg.% (average value of daily random blood sugar levels).

TABLE 3. THE EFFECTS OF INSULIN ON BLOOD SUGAR, GLUCOSURIA, WEIGHT AND PANCREATIC GLYCOGEN INFILTRATION IN ALLOXAN DIABETIC RABBITS: PREVENTION DESPITE HYPERGLYCEMIA

Rabbit number	C50	D48	C91	C49	C89
Duration of treatment (weeks)	6	12	5	16	15
Average daily insulin (units)	5	5	3	4	5
Average blood sugar (mg. %)	413	496	354	488	457
Average glucosuria (gms./day)	23.4	29.4	14.6	16.6	21.3
Change in body weight (kilograms)	+0.49	+1.46	−0.53	+0.74	+2.16
Glycogen infiltration					
islets	some	extreme	none	none	none
ductules	some	extreme	none	none	none
centro-acinar	some	some	none	none	none
Changed insulin dose (units)		9		0	0.8
Duration of changed R (weeks)		3		3	3
Average blood sugar (mg. %)		433		477	606
Average glucosuria (gms./day)		10.1		18.3	37.1
Change in body weight (kilograms)		+0.39		−0.15	−0.77
Glycogen infiltration					
islets		none		some	some
ductules		some		some	some
centro-acinar		none		some	some

The metabolic and histopathologic data are summarized in Table 3.

Rabbit number C50 showed definite widespread deposits of glycogen in the cytoplasm of pancreatic islet, ductular epithelial and centro-acinar cells at autopsy 6 weeks after injection of alloxan. The average blood sugar level was 413 mg.%. Rabbit number D48 had extremely severe and widespread lesions in the various pancreatic cells when examined at biopsy after 12 weeks, during which the average value of daily random blood sugar analyses was 496 mg.%. However, when the daily dose of insulin was

increased slightly, the glycogen disappeared from the islets within three weeks. During these three weeks the blood sugar fell to 433 mg.%.

Number C91 developed a severe infection in and about a joint in the right foreleg. Progression of this inflammatory lesion was not deterred by penicillin therapy. During the fourth and fifth weeks very little insulin was given. At autopsy after five weeks, during which the average blood sugar level was 354 mg. %, no glycogen was demonstrable in the pancreas. Two rabbits, numbers C49 and C89, had average random blood sugar levels of

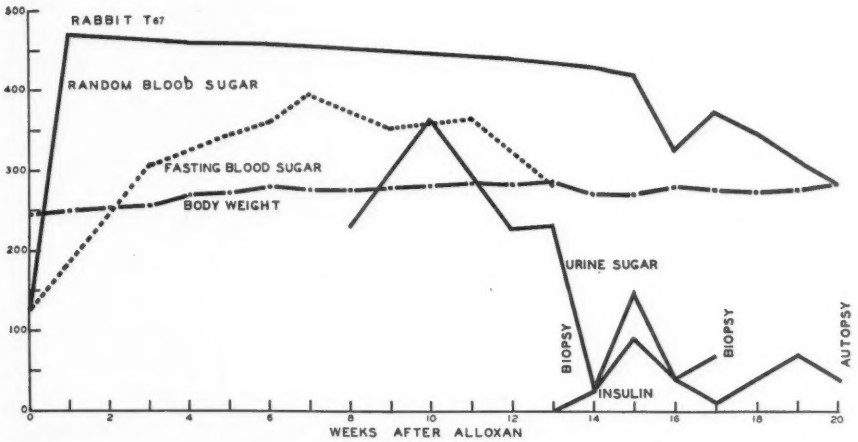

FIG. 3. The metabolic course of alloxan diabetic rabbit number T67 before and during treatment with small doses of insulin. Although marked hyperglycemia persisted during therapy, the extent and severity of glycogen infiltration in the pancreas was much reduced in the second biopsy specimen and the lesion was completely absent in the autopsy sections (see Fig. 4). Note on the left vertical scale: For blood sugar values, 300=*300 mg.%*; for urine sugar values, 300=*30.0 gm. per 24 hours;* for body weight, 300=*3.0 kg.;* and for insulin dose, 50=*5.0 units per day.*

488 and 457 mg.%, respectively, during 4 months of treatment with small doses of insulin given daily. The metabolic course of rabbit number C89 is depicted graphically in Fig. 5. At biopsy neither of the pancreases contained demonstrable glycogen (Fig. 6). After 3 weeks during which no insulin was given to rabbit number C49 and only occasional small doses to rabbit number C89, obvious cytoplasmic accumulations of glycogen appeared in the islets, ductules and centro-acinar cells (Fig. 7). During this terminal three week interval rabbit number C49 showed no significant change in blood sugar level but rabbit number C89 showed a large increment (from 457 to 606 mg.%).

DISCUSSION

These experimental observations corroborate the previous findings of Duff, McMillan and Wilson (1947) with respect to the characteristic occurrence of "hydropic degeneration" in the pancreatic islets and ductules of permanently alloxan diabetic rabbits. The fully developed lesion did not progress to atrophic dissolution of the affected cells nor did the severity of

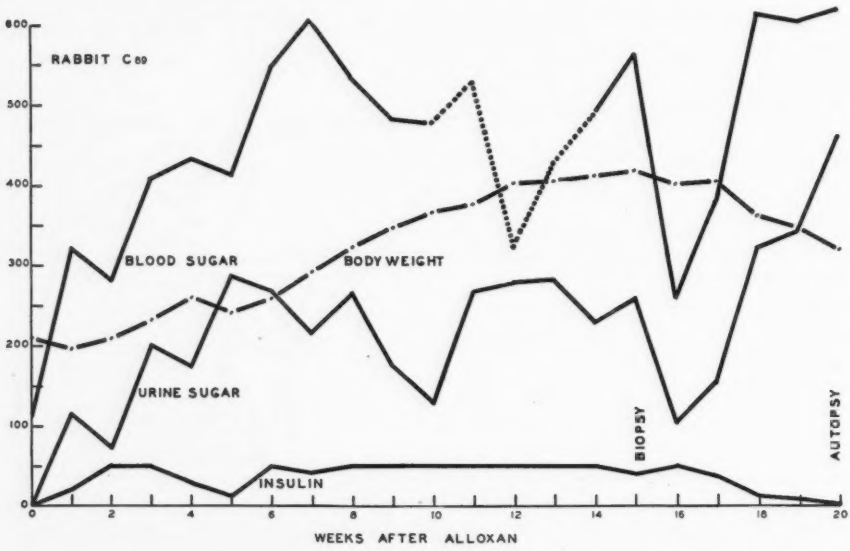

FIG. 5. The metabolic course of alloxan diabetic rabbit number C89 during treatment with small doses of insulin. Although marked hyperglycemia persisted for 4 months, glycogen infiltration was not demonstrable in the pancreatic biopsy. However, when the dose of insulin was reduced, the lesion appeared within 3 weeks (see Figs. 6 and 7). Note on the left vertical scale: For blood sugar values, 300=*300 mg.%*; for urine sugar values, 300=*30.0 gm. per 24 hours;* for body weight 300=*3.00 kg.;* for insulin dose, 50=*5.0 units per day.* The portion of the blood sugar curve indicated by a dotted line represents values determined on single weekly fasting samples.

the diabetic state change appreciably within periods of observation as long as 11 months. Control of diabetic symptoms with large doses of insulin over periods of about 30 days was associated with restoration to normal appearance of the previously affected pancreatic cells. The number of experiments accomplished was insufficient to allow exact determination of minimum dosages and durations of therapy necessary to achievement of this histological result. It was apparent that definite restorative alterations could be found in as short a period as 2 weeks when good control of symp-

toms was attained by giving large doses of insulin. However, when the dose was very small and hyperglycemia of severe degree was continuously present, traces of glycogen persisted in the islets for as long as 29 days. Nevertheless, continuation of such inadequate treatment eliminated all demonstrable deposits of glycogen from the pancreas within a total of 49 days.

Not only could restoration to normal appearance of previously affected pancreatic cells be achieved with small doses of insulin despite hyperglycemia, but also prevention of development of the lesion was possible under similar circumstances. In one experiment (rabbit number C91) it was uncertain whether prevention was attained in this way because the total duration of the experiment was somewhat less than the shortest observed interval between injection of alloxan and development of glycogen infiltration (rabbit number C50, 6 weeks). The average blood sugar levels of rabbits numbers C49 and C89 were so high despite treatment with small doses of insulin that the development of the pancreatic lesion could be confidently expected to have occurred after 4 months, but it did not do so. Furthermore, when the dose of insulin was reduced, both of these rabbits did show the lesion within only 3 weeks even though one of them (rabbit number C49) exhibited no appreciable change in degree of hyperglycemia. Rabbit number D48 showed severe glycogen infiltration in the pancreatic islets and ductules after 12 weeks of treatment with small doses of insulin. When a slightly larger dose was given for three more weeks there was a definite decrease in blood sugar level (from 496 to 433 mg.%) and a marked decrease in glucosuria (from 29.4 to 10.1 grams per day). After this brief additional period of treatment some glycogen remained demonstrable in the pancreatic ductules but none was present in the islet cells. This result is similar to the finding of partial reversal under the influence of small doses of insulin despite continuous, severe hyperglycemia.

The average random blood sugar levels of ten untreated alloxan diabetic rabbits gave a mean value of 530 mg.% and ranged from 390 to 645 mg.%. Of the five rabbits (numbers T23, T20, T64, T67, and D48) that showed reversal subsequent to insulin treatment despite hyperglycemia, all had average random blood sugar levels during treatment that were lower than the mean value for the untreated group. Two of these rabbits (numbers T23 and T67) had values lower than the lower limit of the untreated group. On the other hand, in both instances of prevention with insulin therapy despite hyperglycemia (rabbits numbers C49 and C89), the average random blood sugar levels were lower than the mean value but higher than the lower limit of the values for the untreated group. It would, therefore, appear probable that hyperglycemia played no more than a minor role as a factor in the pathogenesis of glycogen infiltration in the islets of Langerhans, intralobular ductules and centro-acinar cells of the pancreas. It was

apparent that severe, continuous hyperglycemia was quite compatible with complete reversal and prevention of the lesion by very small doses of insulin.

The occurrence of these restorative and preventive phenomena under the influence of small doses of insulin that were quite inadequate to prevent marked hyperglycemia tends to support the original hypothesis of Allen (1913) that a deficiency of hormone in the circulation (i.e. hypoinsulinemia) might provide the stimulus for beta cell hyperfunction. If this were true, one might reasonably expect that a dose of insulin sufficient to prevent or overcome the pancreatic lesions in one diabetic animal might be found ineffective in another, irrespective of the absolute values of their blood sugar levels. Likewise, very different periods of insulin therapy might be found necessary to achievement of restoration of the fully-developed lesions to normal appearance. A simple and accurate method for determining blood insulin levels is needed to establish whether or not the hypothetical beta cell stimulating effect of hypoinsulinemia is a reality.

The source of the affected islet cells would appear to be obscure in view of the common opinion of numerous investigators that one diabetogenic dose of alloxan destroys all, or practically all, of the beta cells in the islets of Langerhans. However, it is apparent that the glycogen-filled islet cells are of beta type, for numerous islet cells having more or less abundant cytoplasmic granules of the beta type were observed repeatedly in the islets of Langerhans of the insulin-treated diabetic rabbits. One can only conclude that considerable numbers of beta cells remain after a diabetogenic dose of alloxan or else that immediate regeneration of beta cells is more active than has been supposed hitherto. The possibility of long continuing regeneration of functioning beta cells seems inconsistent with the long persistence of diabetes, for if this occurred the diabetic state should eventually disappear.

Decreased "islet reserve" caused by the destruction of beta cells in response to alloxan apparently determines failure to secure permanent amelioration of diabetic symptoms by administering a course of insulin therapy. In this respect the alloxan diabetic rabbit seems comparable to the partially depancreatized diabetic dog and to the meta-hypophyseal diabetic dog or cat. In one of our experiments (rabbit number T4) a lesser degree of hyperglycemia was noted in the first few weeks of relapse following discontinuation of insulin therapy. However, no clear evidence of permanent amelioration of the diabetic state referable to insulin treatment was observed in any of our studies.

These studies indicate that treatment with insulin may be responsible, at least in part, for the reported rarity of "hydropic degeneration" in the islets of Langerhans of diabetic human subjects at autopsy. The absence of

the lesion might be anticipated despite prolonged periods of poor therapeutic control, providing that some insulin continued to be given. However, by utilizing several techniques for histological demonstration of glycogen (Toreson, 1950), the existence of the lesion was confirmed in 9 of 11 suspected instances found amongst 26 cases of death in diabetic coma. Glycogen could be demonstrated in the pancreatic islets of one subject who had died in diabetic coma even though terminal over-treatment with insulin had precipitated hypoglycemic convulsions. Terminal hyperglycemia was not, therefore, necessary to the persistence of the lesion, through this short period of time.

SUMMARY

1. Eighteen rabbits, after periods of alloxan diabetes ranging from 8 to 48 weeks, presented in each case glycogen infiltration of the pancreatic islet, ductular and centro-acinar cells (i.e. what has previously been described as "hydropic degeneration" of these cells).

2. Treatment of some of these alloxan diabetic rabbits with large doses of insulin for periods of about 30 days restored normal structural appearance to the pancreatic islet, ductule and centro-acinar cells that had previously become infiltrated with glycogen. The restored islet cells had the cytological characteristics of beta type cells. Upon cessation of therapy symptoms of diabetes reappeared rapidly and glycogen infiltration developed again in the pancreas.

3. Treatment of other alloxan diabetic rabbits with small doses of insulin for periods of several weeks restored glycogen-filled pancreatic cells to normal appearance; this result was attained despite the continuous persistence of marked hyperglycemia during therapy.

4. Treatment of alloxan diabetic rabbits with small doses of insulin for periods of several months beginning immediately after the injection of alloxan prevented the development of glycogen infiltration in the pancreas. Prevention was attained even though hyperglycemia of marked degree persisted continuously during treatment. When therapy was discontinued typical lesions developed more rapidly than was the case when no insulin was given following injection of alloxan. Accelerated development of the lesion occurred regardless of whether or not there was further elevation of the blood sugar level.

5. Clear evidences of permanent amelioration of diabetes or of progressive regeneration of beta cells of the islets of Langerhans were not observed in either the untreated alloxan diabetic rabbits or in those treated with insulin.

6. It appears probable that hypoinsulinemia plays a more important role than does hyperglycemia in the pathogenesis of glycogen infiltration (so-called "hydropic degeneration") in the islets of Langerhans, intra-

lobular ductules and centro-acinar cells of the pancreas of the alloxan diabetic rabbit. Indeed, our own observations yield evidence to indicate that hyperglycemia *per se* has no more than the slightest direct effect.

7. Insulin therapy, even though inadequate to control the symptoms of diabetes, may be partially responsible for the reported rarity of "hydropic degeneration" in the islets of Langerhans of the diabetic human subject.

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THE EFFECT OF ADRENALECTOMY IN THE RAT UPON THE RATE OF GROWTH OF TRANSPLANTABLE TUMORS*

DWIGHT J. INGLE AND BURTON L. BAKER

Research Laboratories, The Upjohn Company, Kalamazoo, Michigan and The Department of Anatomy, University of Michigan School of Medicine, Ann Arbor, Michigan

THE data of these experiments show that adrenalectomy in the rat retards the growth of a transplantable carcinoma and of a lymphosarcoma.

EXPERIMENTAL

Rats of the Sprague-Dawley strain were used in these studies. Experiment 1 involved 42 adult female rats which were maintained on a commercial chow diet. Each rat was inoculated aseptically with the Walker Carcinoma 256. The tumor was macerated, diluted with 0.9 per cent NaCl, and 0.2 cc. of the suspension was injected subcutaneously on the right flank of the rat. As soon as the tumor was palpable (4th or 5th day), the animals were paired so that the weight of each experimental rat did not differ from that of its control by more than 10 gms. At this time, the experimental animals were adrenalectomized and ovariectomized, and the controls were ovariectomized. The food consumption of each control was limited to that of its adrenalectomized mate, the latter being maintained on a 1 per cent solution of NaCl to drink. Seven or 8 days later, the animals were killed and the tumors weighed after being incised to release any fluid contained in them.

Experiment 2 involved 48 male rats having initial weights of approximately 300 gm. These rats were force-fed a medium carbohydrate diet (Ingle, Prestrud and Rice, 1950) throughout the experiment. Following a control period of 14 days, each rat received an implant of the tumor (Walker carcinoma 256) according to the procedure of experiment 1. Four days after inoculation with the tumor, 24 rats were adrenalectomized and 24 were subjected to sham operations. The tumors of all of the animals were excised and weighed on the 7th day following operation.

Experiment 3 involved 25 male rats of approximately 300 gms. initial weight. These rats were force-fed as in experiment 2. After a control period of 14 days, 13 rats were adrenalectomized and 12 were subjected to sham operations. Ten days later, each rat received a small section of a lymphosarcoma (Murphy and Sturm, 1941) by aseptic subcutaneous implantation through a trocar high on the right flank. The tumors were excised and weighed 8 days later.

Experiment 4 involved 10 pairs of rats and was identical with experiment 3 except that the tumors were excised and weighed on the 12th day following implantation.

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RESULTS

The data on averages and standard errors of the averages are summarized in Table 1. The average weights of the tumors were less in the adrenalectomized rats than in the non-adrenalectomized rats in each of the 4 experiments. From the statistical standpoint, the differences in averages are highly significant in both experiments 1 and 2 which involved the Walker carcinoma. The differences in averages for experiments 3 and 4 involving the lymphosarcoma did not meet the usual demands for statistical significance when considered individually, but since the direction of the difference was the same in each experiment, the probability is increased that tumor growth is retarded by adrenalectomy.

TABLE 1. THE EFFECT OF ADRENALECTOMY UPON THE GROWTH OF TRANSPLANTABLE TUMORS IN THE RAT

Experiment	Tumor	Days following operation	Number pairs	Tumor weights gm. Average and standard error	
				adrenalectomy	controls
1	carcinoma	7–8	21	5.25 ±0.57	9.21 ±1.1
2	carcinoma	7	24	6.01 ±0.57	10.62 ±0.97
3	lymphosarcoma	8	12	5.47 ±1.27	7.43 ±1.08
4	lymphosarcoma	12	10	21.1 ±1.87	26.0 ±2.36

DISCUSSION

Only a few data have been published on the rate of tumor growth in adrenally insufficient animals. Laclau (1930), Bischoff, Maxwell and Ullmann (1931) and Bischoff and Maxwell (1932) found no effect of adrenalectomy upon the incidence of takes or the growth of tumors in rats. Roffo (1930) reported that adrenalectomy in the rat markedly reduced the incidence of takes of transplantable sarcoma and adenocarcinoma. Sturm and Murphy (1944) demonstrated that the adrenalectomized rat had a lower than normal resistance to a transplantable lymphatic leukemia. Joannovics (1916) implanted control and adrenalectomized mice with carcinoma, sarcoma and chondroma tumors. There was no effect of adrenalectomy upon the number of takes, but significant inhibition of growth of the sarcoma and chondroma was reported.

We are interested in the effect of either adrenal cortical insufficiency or excess upon the growth of both normal and abnormal tissues. Tumors are of interest because of their very rapid rate of anabolism. Ingle (1950) has discussed the relationship between adrenal cortical function and growth. Growth of both normal (Baker, Ingle, Li and Evans, 1948; Baker and Ingle, 1948; Baker and Whitaker, 1950) and abnormal tissues can be suppressed by hypercorticalism. The administration of large doses of

cortisone retards the rate of growth of both the Walker carcinoma 256 (Ingle, Prestrud and Rice, 1950) and the Murphy-Sturm lymphosarcoma (Ingle, in press). It is also evident that rapidly growing tissues cannot attain a peak rate of anabolism in adrenally insufficient animals. This is true for somatic growth (Ingle and Prestrud, 1949), and the data of the present experiments show that it is true of the Walker carcinoma and is probably true of the Murphy-Sturm lymphosarcoma. The essentiality of the adrenal cortical hormones for optimal growth may be non-specific rather than reflecting any specific anabolic action of these hormones.

SUMMARY

In 4 separate experiments, one rat of each pair was adrenalectomized, and the remaining animals were subjected to control operations. In experiment 1, 42 adult female ovariectomized rats were hosts to the Walker carcinoma 256. Following operation, the rats were paired-fed. In experiment 2, 48 force-fed male rats were hosts to the same tumor. The weights of the tumors were determined 7 days following operation, and in both experiments, adrenalectomy significantly retarded the rate of tumor growth. In experiments 3 and 4, force-fed male rats were hosts to the Murphy-Sturm lymphosarcoma. Twenty-five rats were studied in experiment 3, and the tumors were weighed 8 days following operation. Twenty rats were studied in experiment 4, and the tumors were weighed 12 days following operations. In each group, the average weight of the tumors from adrenalectomized rats was less than for the controls, but the differences did not fully meet the usual requirements for statistical reliability.

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A METHOD OF ASSAYING ADRENAL CORTICAL HORMONES BASED ON A DECREASE IN THE CIRCULATING EOSINOPHIL CELLS OF ADRENALECTOMIZED MICE[1]

ROBERT S. SPEIRS[2] AND ROLAND K. MEYER

Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine and the Department of Zoology, University of Wisconsin

THERE is a growing demand at the present time for a relatively quick bioassay procedure for the 11-oxycorticosteroid hormones of the adrenal cortex. Since 1930 numerous assay procedures have been proposed, based upon survival, growth, resistance or glycogen deposition of adrenalectomized animals, as well as specific target responses such as the muscle work test. These procedures have recently been compared (Thayer 1946; Dorfman 1949; Sayers 1950). However, the numerous modifications of each procedure and the general lack of agreement on any one procedure clearly indicate that a satisfactory assay has not yet been found and accepted.

The following paper includes some of the data and results accumulated over a period of 2½ years. These results led the authors to propose an assay procedure in adrenalectomized mice based upon a decrease in the number of circulating eosinophil cells following a subcutaneous injection of an adrenal cortical steroid (Speirs and Meyer, 1949 and 1950). This procedure appears to be highly sensitive, specific, rapid and relatively easy to perform. With proper guidance, the techniques of doing accurate blood counts can be mastered in 2 to 3 weeks by experienced laboratory technicians. A quantitative assay and the necessary calculations may be performed within 12 hours. The mice may be reused many times, thereby eliminating the necessity for large colonies of animals.

MATERIALS AND METHODS

The procedures for obtaining blood samples from mice and performing eosinophil counts have been published earlier (Speirs and Meyer, 1949). The animals were care-

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fully warmed and blood samples were taken from the tail within 10 minutes from the time they were first disturbed. All eosinophil counts published in this report represent actual numbers of cells counted in two Fuchs-Rosenthal Chambers (a total of 32, 1 square millimeter areas). It was not necessary to convert these figures to determine the number of cells per millimeter of blood, but this can easily be done by multiplying the number of cells counted by 3.1. The standard error of this procedure was determined by taking multiple samples of blood from the same mouse, determining the eosinophil count, and calculating the variation between counts. In our laboratories, the standard error of a single eosinophil count was found to be 1.2 times the square root of the number of cells counted.

Male Jax C57 Brown mice, subline cd, weighing 20 to 25 grams were used in this study. All the mice were adrenalectomized in a one step operation and maintained at 28°C. with 1% sodium chloride added to their drinking water. In the early experiments blood determinations were made only twice on each animal (3 and 10 days postoperatively). Later it was found that by implanting 15 milligram desoxycorticosterone acetate pellets and by pretreating adrenalectomized mice with epinephrine, as described in this paper, it was possible to re-use the animals many times.

Compound F and cortisone (free alcohol) were dissolved in water and added to propylene glycol or glycerine. Cortisone acetate was dissolved in benzyl alcohol and then mixed with sesame oil in proportions of 1 to 10. Other steroids were dissolved in ether, which was added to sesame oil, and then heated to evaporate the ether.

PROCEDURES AND RESULTS

Experiments were designed to determine the dose-response of adrenalectomized mice to a commercial extract of the adrenal cortex (Lipo-Adrenal Cortex, Upjohn), to synthesized 11-oxycorticosteroid hormones (cortisone and compound F), and to various control solutions. A single injection of these materials was made 3 and 10 days after adrenalectomy and blood samples were taken immediately before and 4 hours following the injection. Table A summarizes the per cent decrease in circulating eosinophils.

The larger doses, (0.005 to 0.008 cc equivalent of Lipo-Adrenal Cortex or 6 micrograms of Cortisone acetate or compound F), produced an average decrease of 80% or more in the number of circulating eosinophils. Lower doses produced smaller mean decreases. However, it will be noted that even in very low doses (0.0005 to 0.002 cc of Lipo-Adrenal Cortex or 0.25 and 0.5 microgram of the 11-oxycorticosteroids) some of the animals gave responses up to 85% decrease. In the control series, 24 mice receiving no injections or injections of corn oil, showed only slight to moderate decreases in eosinophils. In this series, one mouse showed a decrease of 38%, six others decreased slightly, and 17 showed no decrease during the 4 hour period. However, in the control animals which were injected with a 10% benzyl alcohol solution in sesame oil, a mean decrease of 45% was obtained. This decrease occurred in 23 out of the 24 animals. The benzyl alcohol was slightly toxic to the animals, and it is believed that this toxicity

was a non-specific stimulus which produced a decrease in the number of circulating eosinophils.

In summary, a decrease in the number of circulating eosinophils could be correlated with graduated doses of adrenal cortical hormones. However, in some of the animals receiving injections of control or very dilute hormone solutions, a non-specific decrease in eosinophils occurred. This non-

TABLE A. PER CENT DECREASE IN CIRCULATING EOSINOPHILS FOLLOWING A SUBCUTANEOUS INJECTION OF VARIOUS ADRENAL CORTICAL MATERIALS INTO ADRENALECTOMIZED MICE

Injection		Number of animals	Pretreatment	Per cent decrease in eosinophils			S.D.	S.E.
Material	Dose*			Mean	Maximum response	Minimum response		
Lipo-Adrenal Cortex (Upjohn)	0.008 cc.	9	None	86	100	39	15	5.2
	0.007	7		88	96	77	7	2.7
	0.006	8		84	93	67	8	2.9
	0.005	8		83	87	69	7	2.6
	0.004	14		73	90	61	9	2.3
	0.003	19		77	95	54	14	3.1
	0.002	13		49	83	0	33	9.1
	0.001	25		47	85	0	28	5.6
	0.0005	21		36	82	0	27	6.0
		128 Mice						
Compound F (17 hydroxy-cortico-sterone)	6 mμ	9	None	80	95	14	26	8.5
	3 mμ	9		61	74	39	15	4.8
	1 mμ	9		37	63	0	23	7.7
	0.5 mμ	9		14	49	0	20	6.6
		36 Mice						
Cortisone Acetate	6 mμ	8	None	81	92	68	10	3.8
	6 mμ	18		72	94	0	22	5.1
	1 mμ	24		56	87	0	23	4.6
	0.5 mμ	17		42	70	0	24	5.8
	0.25 mμ	12		46	78	0	26	7.4
		79 Mice						
Controls Non-injected	—	14	None	5	23	0	9	2.4
Corn Oil	0.03 cc.	10		10	38	0	16	5.2
10% Benzyl alcohol in Sesame oil	0.03 cc.	24		45	88	0	24	4.9
		48 Mice						

* The material was added to sesame oil so that a constant injection volume of 0.03 cc. was used.

specific response was greatest following injections of toxic materials such as 10% benzyl alcohol and is believed to be due to release of hormone from adrenal cortical rests (Speirs and Meyer, 1949 and 1950). Thus in an assay procedure involving a decrease in eosinophils in adrenalectomized mice, one would have to consider the possibility of a non-specific response, especially when toxic materials are injected.

It has been found that a single injection of epinephrine will render the eosinophils of adrenalectomized mice refractory to further injections of epinephrine for a period of 4 hours (Speirs and Meyer, 1950). This has also

been found by Recant, *et al.* (1950) in rats, dogs and humans. Therefore, a series of experiments were designed to determine the dose-response of eosinophils to adrenal cortical materials in epinephrine-pretreated adrenal. ectomized mice. These mice received adrenal cortical hormones during

TABLE B. PER CENT DECREASE IN CIRCULATING EOSINOPHILS FOLLOWING A SUBCUTANEOUS INJECTION OF VARIOUS ADRENAL CORTICAL MATERIALS INTO EPINEPHRINE-PRETREATED ADRENALECTOMIZED MICE

Injection		Number of animals	Pretreatment	Per cent decrease in eosinophils			S.D.	S.E.
Material	Dose*			Mean	Maximum response	Minimum response		
Lipo-Adrenal Cortex (Upjohn)	0.03 cc.	8	3–5 micrograms Epinephrine	96	100	81	6	0.7
	0.015	7		86	94	80	5	1.9
	0.008	9		67	88	44	15	8.9
	0.004	10		69	86	33	20	6.6
	0.003	11		64	93	28	20	6.2
	0.002	13		32	75	0	26	3.6
	0.0015	10		24	57	0	21	6.5
	0.0010	8		9	39	0	17	6.2
	0.0005	2		0	0	0	0	0
		78 mice						
Compound F	6 mμ	23	3–5 mμ Epinephrine	79	96	56	11	2.2
	3	24		60	84	0	26	5.0
	1	14		13	58	0	19	5.2
		61 mice						
Cortisone Acetate	96 mμ	3	5 mμ Epinephrine	95	99	87	6	3.8
	24	3		92	94	91	2	1.2
	12	21		77	94	57	10	2.5
	6	23		78	94	43	13	2.7
	3	21		53	88	21	19	4.2
	1	21		18	51	0	21	4.7
	0.5	6		7	28	0	10	4.1
		98 mice						
Controls, No 2nd Injection	—	15	5 mμ	1	18	0	5	1.2
Sesame Oil	0.03 cc.	10		3	24	0	8	2.4
10% Benzyl alcohol in Sesame oil	0.03 cc.	10		2	10	0	10	3.1
		35 mice						

* The material was added to sesame oil so that a constant injection volume of 0.03 cc. was used.

a period in which they were refractory to epinephrine and presumably to other toxic materials.

In this experiment the adrenalectomized mice received a subcutaneous injection of 3 to 5 micrograms of epinephrine in the caudal region of the back and 4 hours later the adrenal cortical hormones were injected into the cervical region. The first eosinophil count was taken immediately prior to the injection of the adrenal cortical hormone and a second count was taken 3 hours later. The results, summarized in Table B, show a graded response to progressively decreasing doses of adrenal cortical hormones. This gradation in response is particularly noticeable in the lower doses such

as 0.004 to 0.001 cc of Lipo-Adrenal Cortex and 1 to 6 micrograms of cortisone acetate or compound F. Very low doses and control solutions had only a slight, insignificant effect upon the eosinophils. The group receiving the 10% benzyl alcohol solution showed a mean decrease of 2%. This can be compared with the 45% mean decrease received in the animals without

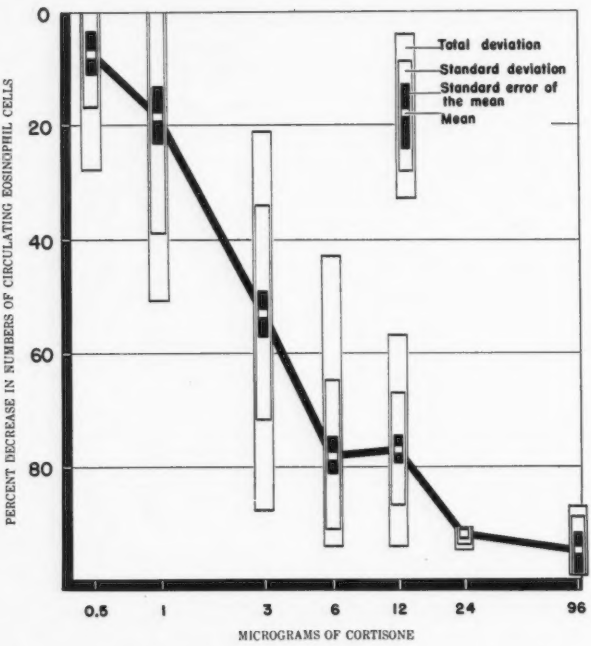

FIG. 1. The Log-Dose-Response to cortisone acetate of circulating eosinophils in epinephrine-pretreated adrenalectomized mice.

pretreatment. It is evident therefore that pretreatment with epinephrine reduces or eliminates the non-specific decrease in eosinophils.

The log-dose-response of the eosinophils of pretreated adrenalectomized mice to cortisone acetate is plotted in Figure 1. It can be seen that between 0.5 and 6 gamma there is approximately a straight line dose-response relationship.

Further experiments were carried out in pretreated adrenalectomized mice using other adrenal hormones and non-specific materials and the results are tabulated in Table C. 11-Desoxycorticosterone acetate in doses of 100 micrograms or more produced a decrease in the number of eosinophils. However, this was not a graded response. Low doses (30 to 50 micro-

grams) produced no significant decrease in the eosinophils. Higher doses (100 to 400 micrograms) produced less than a 50% mean decrease in the circulating eosinophils. Cortisone (free alcohol) dissolved in water and injected subcutaneously produced a dose-response curve almost identical

TABLE C. EOSINOPENIC ACTIVITY OF VARIOUS MATERIALS INJECTED INTO EPINEPHRINE-PRETREATED ADRENALECTOMIZED MICE

Injection material	Dose	Number of mice	Per cent decrease in eosinophils		
			Mean	Maximum response	Minimum response
11-Desoxycorticosterone Acetate (Ciba)	400 mμ	5	42	63	15
	200	5	47	48	43
	100	5	49	80	3
	50	5	9	19	0
	30	5	8	38	0
		25 Mice			
Cortisone Free Alcohol (Merck) (Water Soluble)	6 mμ	6	81	85	77
	3	6	44	77	2
	1	6	24	64	0
	0.5	5	4	19	0
	Controls	6	0	0	0
		29 Mice			
Compound A (Dehydrocorticosterone)	25mμ	5	72	84	52
Compound G (Kendall)	12mμ	8	0	0	0
Androsterone	100	6	0	0	0
Testosterone	100	4	0	0	0
Dehydro-iso-androsterone	100	3	0	0	0
Progesterone	100	5	0	0	0
Alpha estradiol	50	3	0	0	0
Δ^5-3 ethinyl adrostene-3, 17-Diol	300	4	2	8	0
Δ^4-adrostene-3,17-dione	300	3	0	0	0
Δ^5-3-hydroxy pregnenone-20	300	3	0	0	0
Δ^5-3-acetoxy-ethiocholenic Acid	300	6	2	11	0
Δ^4-pregnene-17 (a), 20 (b), 21,-triol-3-one	300	3	17	35	0
3,21-diacetoxy-11,20-diketo-pregnane	300	6	15	45	0
3,21 dihydroxy-11-keto-Δ^{17}-20-cyano pregnene	300	3	0	0	0

to that obtained with cortisone acetate dissolved in benzyl alcohol and sesame oil (see Figure 1). Compound A in 25 microgram doses, had an eosinopenic effect. Various sex hormones and non-specific steroids in 50 to 300 microgram doses had little or no eosinopenic effect. In a separate series of experiments over 60 steroids were injected into pretreated, adrenalectomized mice and the results indicate that only adrenal cortical steroids with an oxygen at C 11 and a double bond at C4–5 will produce a marked

per cent decrease in eosinophils. With the exception of 11-desoxycorticosterone acetate (which produces only a partial response) all other compounds tested to date had little or no eosinopenic effect.

Additional experiments were designed to determine whether eosinopenic materials are present in urine. A 10 cubic centimeter sample was taken from pooled human pregnancy urine. One cubic centimeter of the untreated urine was injected subcutaneously into epinephrine-pretreated adrenalectomized mice. The urine was also diluted with varying quantities of 0.9% saline solution as shown in Table D and injected in the same manner to determine whether a graded response could be obtained. It may be seen that the per cent decrease in eosinophils varied inversely with the

TABLE D. EOSINOPENIC ACTIVITY OF UNTREATED PREGNANCY URINE IN EPINEPHRINE-PRETREATED ADRENALECTOMIZED MICE

Volume Injected	Dilution	Number of animals	Pretreatment	Decrease in eosinophils		
				Mean	Maximum	Minimum
1 cc.	Undiluted	5	5 mμ Epinephrine	80%	91%	74%
1 cc.	1/2.5	6		80%	88%	74%
1 cc.	1/10	6		61%	70%	39%
1 cc.	1/20	6		32%	73%	3%
1 cc.	1/40	6		25%	49%	0
1 cc.	Saline	10		8%	30%	0

dilution of the urine. One cubic centimeter injections of saline produced a decrease in half of the mice; however, the amount of decrease averaged only 8%. Thus eosinopenic materials are present in untreated pregnancy urine in large enough quantities to be easily assayed by this procedure. On the other hand, a urine sample from a patient with rheumatoid arthritis produced only a slight eosinopenic effect when injected into pretreated adrenalectomized mice.

The log-dose response of the per cent decrease of eosinophils to various adrenal cortical materials and urine is plotted in Figure 2. The slopes of these curves show no significant variation from that obtained following injections of cortisone acetate. Furthermore, the eosinopenic activities of compound F, cortisone acetate, and cortisone (free alcohol) are very much alike.

DISCUSSION OF RESULTS

It may be seen from the data presented in this paper that the change in number of circulating eosinophils can be used as a sensitive assay procedure for relatively small quantities of adrenal cortical materials. In general, this procedure has been found to be rapid, easy to perform, extremely sensitive, and relatively accurate when performed by experi-

enced technicians. Six micrograms of cortisone or compound F will produce a maximum decrease (80% or more) in the numbers of circulating eosinophils of adrenalectomized mice. In animals pretreated with epinephrine as little as one microgram of these materials will produce a noticeable decrease. A complete quantitative assay can be performed with fewer than 20 mice (3 doses, 6 mice per dose), and as little as 60 micrograms of material. Moreover, the mice may be re-used each week for additional assays.

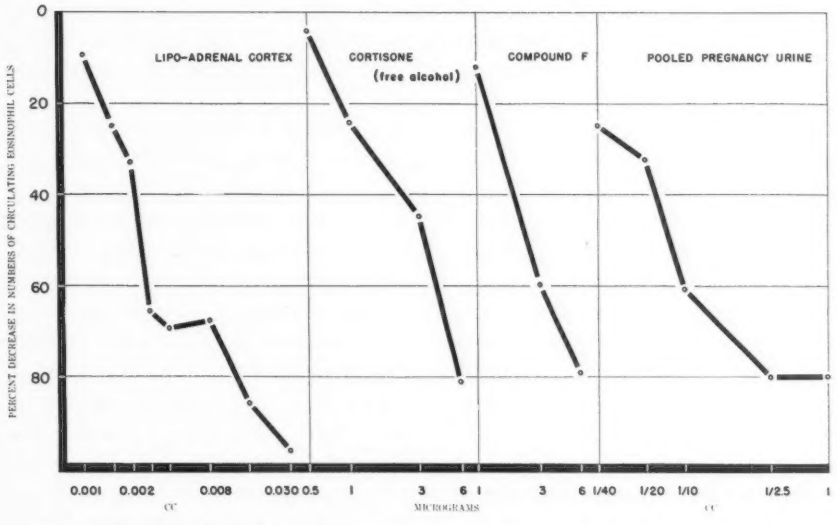

FIG. 2. The Log-Dose-Response to various materials of circulating eosinophils in epinephrine-pretreated adrenalectomized mice.

Dorfman (1949) showed that the liver-glycogen deposition test in fasting adrenalectomized rats was the most accurate method for assaying adrenal cortical materials at that time. However, large numbers of animals as well as large quantities of hormone (minimum 250 micrograms per rat) were needed. The liver glycogen deposition test in adrenalectomized mice was 25 times more sensitive, but a decrease in precision occurred. For this procedure the minimum amount of cortisone required per animal was 10 micrograms, or between 100 and 400 micrograms per assay. Moreover, the procedure was rather lengthy involving many steps over a period of several days, and the animals were sacrificed.

For the above reasons, the authors believe that the techniques proposed in this paper may be preferable for laboratory and clinical use in assaying adrenal cortical materials.

In setting up the eosinopenic method for assaying adrenal cortical materials, it has been found that many precautions must be taken in order to obtain accurate data and results. For example the strain of mice used in the assays is very important. In our experiments the Jax C57 Black mice proved to be as sensitive to cortisone as the Jax C57 Browns reported in this paper, but their black color makes it difficult to locate the tail blood vessels. On the other hand, 5 other strains of mice which were tested proved to have a markedly reduced sensitivity to cortisone, and thus are unsatisfactory for use as assay animals unless large amounts of material are available. Also any mice in poor physical condition or those with low eosinophil counts (less than 100 eosinophils per cubic millimeter of blood) are not satisfactory.

The dose of epinephrine used in these experiments with C57 Brown mice was 3–5 micrograms or approximately 0.2 mg. per kilogram body weight. In other strains of mice a pretreatment of as much as 1 mgm. per kilogram was necessary in order to eliminate the non-specific response. In general the dose of epinephrine should be as high as the animals can tolerate without showing signs of shock or extreme irritation.

The temperature at which the mice are maintained also seems to be important. This problem was solved at the Jackson Laboratory by building a cabinet which maintains the mice in the dark at 28°C. This has proved to be a convenient apparatus in which to store animals before and during an assay.

The amount of error obtained in an eosinophil count can be very great if the prescribed techniques have been varied. Therefore, before routine assays are performed by new assistants, or where local laboratory conditions impose changes in the techniques, it is advisable to check errors of the procedure by making multiple eosinophil determinations from the same mouse and calculating the variation. In our laboratories the standard error of a single count was found to be 1.2 times the square root of the number of cells counted. However, great variation occurred whenever the blood was not free-flowing and the tail had to be manipulated to express sufficient blood. We also used additional precautions to eliminate subjective errors by devising special methods for recording data.

Implantations of pellets of 11-desoxycorticosterone acetate may be made at the time of adrenalectomy. These pellets do not interfere with the specificity or sensitivity of the assay, but they do maintain the animals in better physical condition. With the help of these pellets the animals may be re-used many times. In our experiments the adrenalectomized mice with pellets of DCA were used once a week, and then discarded when the initial count of eosinophils was less than 100 cells per cubic millimeter of blood.

It was found that a maximum decrease in eosinophils does not occur until 4 hours after a moderately large injection of cortisone. However, after injecting small doses of the hormone the maximum response seems to occur in slightly less than 4 hours followed by rapid recovery (5 hours). In order to standardize the procedure and to allow sufficient time for handling large numbers of animals, the second eosinophil count was made 3 hours following the injection of the adrenal cortical material. Thus the time intervals are as follows: the mice are injected with epinephrine and 4 hours later a blood sample is taken for an eosinophil count. Immediately following this the mice are injected with the cortisone or material to be assayed, and 3 hours later a second blood sample is taken.

SUMMARY AND CONCLUSIONS

A method of assaying adrenal cortical hormones has been described. This assay is based on a decrease in the number of circulating eosinophils in adrenalectomized mice during a 3 hour period following a subcutaneous injection of an 11-oxycorticosteroid hormone. In some animals, presumably mice with adrenal cortical rests, a non-specific response was obtained. This occurred primarily following injections of toxic materials such as benzyl alcohol. The non-specific response was eliminated by treating the adrenalectomized mice with epinephrine prior to the adrenal cortical hormone injection.

The assay is performed as follows:

1. Jax C57 Brown mice weighing 20 to 25 grams are adrenalectomized in a one step operation and 15 mgm pellets of 11-desoxycorticosterone acetate are implanted subcutaneously.
2. Three days postoperatively the mice receive a subcutaneous injection of 5 micrograms of epinephrine and the material to be assayed is injected 4 hours later.
3. Eosinophil counts are taken immediately prior to and 3 hours following the injection of the assay material. The per cent decrease of the number of eosinophils is correlated with the quantity of 11-oxycorticosteroid hormones injected.

Using this assay, a straight line log-dose-response was obtained between 0.5 and 6 micrograms of cortisone acetate, cortisone (free alcohol) and compound F. Similar dose responses were obtained with a commercial extract of the adrenal cortex and also with untreated, pooled pregnancy urine. Androsterone, testosterone, estradiol, progesterone and other non-adrenal steroids in the doses injected had no eosinopenic effect. 11-Desoxycorticosterone acetate produced a partial eosinopenic response when relatively large doses (over 100 micrograms) were given.

The authors would like to express appreciation for the splendid technical assistance of Kenneth E. Shaw, Martha Durgin, and James E. Sullivan. The Ciba Pharmaceutical Company kindly furnished hundreds of desoxycorticosterone acetate pellets used in these experiments. The Lipo-Adrenal Cortex and compound F were supplied to Dr. R. K. Meyer by the Upjohn Company. Merck & Company supplied many of the other steroids used. Much of the data presented in this paper was repeated and confirmed by summer students of the R. B. Jackson Memorial Laboratory during the summer of 1950.

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THE EFFECT OF HYPOPHYSECTOMY ON THE UPTAKE OF RADIOACTIVE IODINE[1] BY THE THYROID OF THE RAT[2]

RAYMOND V. RANDALL AND A. ALBERT

From the Mayo Foundation and Mayo Clinic, Rochester, Minnesota

SUBSEQUENT to the initial use of radioactive iodine in the study of the physiology of the thyroid by Hertz, Roberts and Evans (1938), many investigators have demonstrated by means of I^{131} that the administration of thyrotropic hormone augments the avidity of the thyroid of the normal animal for iodine. On the other hand, little information has been forthcoming about the capacity of the thyroid to collect iodine in the absence of thyrotropic hormone resulting from hypophysectomy. LeBlond, Sue and Chamorro (1940) showed that following hypophysectomy the thyroid is no longer capable of collecting as much radioactive iodine as the thyroid of a normal animal. This was later confirmed by Leblond and Sue (1941), Morton and co-workers (1942), Cortell and Rawson (1944) and Leblond and Gross (1948). However, the magnitude of the defect in the collection of iodine following hypophysectomy and the time necessary for the defect to become evident are not clear from these studies. The present study was undertaken in an effort to clarify the temporal relationship between hypophysectomy and its effect on the collection of radioactive iodine by the thyroid of the rat.

MATERIALS AND METHODS

Male rats of the Sprague-Dawley strain were used. They were kept at room temperature (23°C. ± 2°) in metal cages with screen flooring to prevent coprophagia. At the age of 4 weeks all animals were given free access to a Remington iodine-deficient diet number 347 (Remington, 1937) with distilled water and were maintained on this regimen for two to four weeks before being used for experimental purposes. Throughout the course of the experiment the only dietary change was after hypophysectomy had been performed in some of the animals. At that time 5 per cent glucose in distilled water was permanently substituted for the distilled water in the diets of both the hypophysectomized animals and the nonhypophysectomized controls.

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[1] The radioactive iodine used in this study was obtained from the Isotopes Division of the Oak Ridge National Laboratory of the Atomic Energy Commission, Oak Ridge, Tennessee.

[2] Abridgment of part of thesis submitted by Dr. Randall to the Faculty of the Graduate School of the University of Minnesota in partial fulfillment of the requirements for the degree of Master of Science in Medicine.

Each tracer dose contained 1.0 microcurie I^{131} and was injected subcutaneously into the tissues of the back of each rat by means of a 1.0 cc. tuberculin syringe. Since each animal was to receive several tracer doses of I^{131} during the experiment, accurate comparison of the uptake curves could not be made unless each dose contained the same amount of I^{131}. Maximal accuracy of dosage was ensured by making a stock solution of I^{131} from which the tracer doses were withdrawn and injected directly into the test animals without further diluting or pipetting. The initial concentration of radioactivity in the stock solution and the rate of physical decay being known, it was possible to calculate what volume of solution contained 1.0 microcurie of radioactivity each time tracer doses were withdrawn. The diluent used in making the stock solution was a solution containing 100 micrograms NaI, 10 mg. $NaHSO_3$ and 10 mg. NaOH per liter of distilled water. The use of this solution effectively presented the adsorption of the I^{131} to the stock bottle and the syringe used for injections. Essentially carrier-free I^{131} was diluted with this solution in such a manner that each tracer dose of 1.0 microcurie of I^{131} was contained in 0.3 to 1.0 cc. of solution, the exact volume being determined by the physical decay of the I^{131}.

The uptake of I^{131} by the thyroid of each rat was determined by measuring the amount of I^{131} in the thyroid at approximately two, four, seven, twelve and twenty-four hours after the injection of the tracer dose. All measurements were made with an *in vivo* gamma ray counter, described by Albert (1950). Thus, the same animal could be used for several phases of the study. After injection, measurements were made on each animal at varying intervals during the first twenty-four hours. These measurements were corrected appropriately both for the physical decay of I^{131} and for variations, if any, in the counting efficiency of the tube. Ordinarily the latter correction was not necessary, since measurement of a sample of I^{131} which was made throughout the entire series of measurements disclosed a variation of less than 2 per cent.

When the animals were reinjected at some time following the initial dose of I^{131} it was necessary to correct all subsequent measurements for the residual I^{131} left in the thyroid from the previous injection. This was done by measuring the residual radioactivity immediately before the additional tracer dose was injected. The value thus obtained was appropriately corrected for physical decay, biologic decay (v. i.) and the counting efficiency of the tube and subtracted from measurements made while following the uptake of this additional tracer dose. The correction for biologic decay in normal rats was determined to be 0.36 per cent per hour. In the case of hypophysectomized rats, it was found to be negligible and therefore has been omitted in our calculations (Randall, 1950).

The uptake curve of I^{131} by the thyroid of each animal after administration of a tracer dose was constructed by plotting on arithmetic graph paper the several net measurements obtained as described previously and expressed as counts per second versus time in hours. The asymptote for each curve was located and expressed in counts per second. If the curve did not approach an asymptote within the period of observation or if an asymptote could not be constructed the highest point on the curve was considered to be the asymptote. Subsequent uptake curves in the same animal were compared with the first curve (control curve) by means of the formula:

$$\frac{\text{Asymptote of subsequent curve}}{\text{Asymptote of control curve}} \times 100 = \text{Uptake (in per cent of control curve).}$$

RESULTS

Experiment 1. The effect of hypophysectomy on the uptake of I^{131} administered at varying intervals after operation.—Sixteen normal rats were each

injected with 1.0 microcurie of I^{131}. The uptake curve of I^{131} by the thyroid of each animal was determined according to the procedure discussed in the previous section and designated the control curve. Twenty-four hours after the injection of the I^{131}, the pituitary of each animal was removed through the parapharyngeal approach.[3] The rats were then divided into three groups and given additional injections of 1.0 microcurie of I^{131}. The uptake curves were determined for each animal and compared with the control curve for that same animal. These data are condensed into Table 1.

The first group consisted of 4 rats. One day after hypophysectomy the mean of the uptake curves was 61.9 per cent of the control curves. The

TABLE 1. EFFECT OF HYPOPHYSECTOMY ON THE UPTAKE OF I^{131} ADMINISTERED AT VARYING INTERVALS AFTER OPERATION

Group	Number of animals	Mean uptake and range, in per cent of control						
		Control	1*	2	3	4	7	13
I	4	100	61.9 (41.5–78.6)					
II	5	100		28.9 (16.8–42.4)		13.8 (5.3–27.3)		
III	7	100			11.0 (7.5–17.6)		7.0 (2.9–15.3)	8.6 (7.0–13.0)

* Number of days after hypophysectomy.

second group consisted of 5 rats. At two and four days after hypophysectomy, the mean of the uptake curves was 28.9 and 13.8 per cent of the control curves, respectively. The third group consisted of 7 rats. At three, seven and thirteen days after hypophysectomy, the mean of the uptake curves was 11.0, 7.0 and 8.6 per cent of the control curves, respectively.

A composite curve of these findings is presented in Figure 1. Between five and six days after hypophysectomy the thyroidal uptake of I^{131} reached a minimal value of about 8 per cent of normal and remained at this level throughout the subsequent period of observation.

Experiment 2. The effect of repeated doses of I^{131} on the uptake of I^{131} by nonhypophysectomized rats.—As a control for the animals in experiment 1, 13 normal rats were each injected with 1.0 microcurie of I^{131}, and the uptake curve of I^{131} by the thyroid of each animal was determined. The animals were then divided into two groups and given additional injections of I^{131}. The uptake curves were determined for each animal and compared with the control curve for that same animal. These data are condensed into Table 2.

[3] At the end of the experiment, the completeness of hypophysectomy in each animal was checked by microdissection. When hypophysectomy was found to be incomplete all data on the animal in question were discarded.

The first group consisted of 6 rats. At three, five and nine days after the first dose of I^{131}, the mean of the uptake curves was 86.7, 77.4 and 63.9 per cent of the control curves, respectively. The second group consisted of 7 rats. At four, eight and fourteen days after the first dose of I^{131}, the

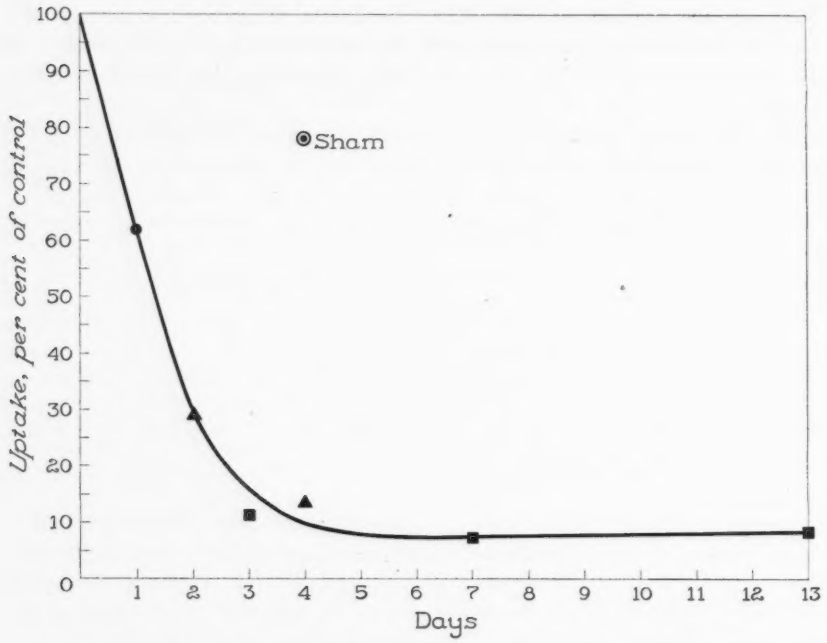

FIG. 1. The effect of hypophysectomy on the thyroidal uptake of I^{131} at varying intervals after operation.

mean of the uptake curves was 76.6, 83.1 and 81.1 per cent of the control curves, respectively. It is evident that at all time intervals the uptake of I^{131} by the thyroid of the nonhypophysectomized animal was always considerably greater than that of the hypophysectomized animal.

TABLE 2. UPTAKE OF REPEATED DOSES OF I^{131} BY NONHYPOPHYSECTOMIZED RATS

Group	Number of animals	Mean uptake and range, in per cent of control						
		Control	3*	4	5	8	9	14
I	6	100	86.7 (70.3–110.0)		77.4 (56.3–96.8)		63.9 (50.0–72.3)	
II	7	100		76.6 (70.2–80.7)		83.1 (54.3–96.6)		81.1 (77.8–87.9)

* Number of days after the first injection of I^{131}.

Experiment 3. The effect of sham-hypophysectomy on the uptake of I^{131}.—As mentioned previously, the parapharyngeal approach was used in removing the pituitaries of the hypophysectomized animals employed in this study. It was impossible to perform this operation without disturbing the thyroid because, in each instance, the incision passed near the thyroid area and the trachea was retracted during most of the procedure. To rule out the possibility of trauma to the thyroid or a nonspecific effect of trauma to the organism being responsible for the depression in uptake of I^{131} by the thyroid noted after hypophysectomy, a sham-hypophysectomy was performed on 4 rats after a control thyroidal uptake curve of I^{131} had been established for each animal. Four days after operation, each animal was given an additional injection of 1.0 microcurie of I^{131} and its uptake by the thyroid was followed. The mean of the uptake curves was 78.3 per cent of the control curves with a range of 73.3 to 88.4 per cent. This value is well above the mean of 13 per cent observed for hypophysectomized rats at four days after operation (Fig. 1) and is within the range of the expected uptake by nonhypophysectomized animals.

DISCUSSION

By the techniques used in this study, the hypophysectomized rats, after the fifth day following operation, continued to collect radioactive iodine in amounts that were about 8 per cent of that taken up before operation. Allowing for the depression of some 25 per cent in the control rats, the defect in I^{131} collection would amount to about 12 per cent of that of normal animals at the end of one week following loss of the pituitary. The cumulative uptake curves of the individual tracer doses by both hypophysectomized and normal animals are similar in shape so that the difference in magnitude of collection in hypophysectomized rats is probably due both to the decrease in trapping function (Vanderlaan and Greer, 1950) and to the organic binding mechanism (Chaikoff and Taurog, 1949).

It is not clear why the thyroids of the normal rats that were given several doses of I^{131} collected smaller amounts of radioactivity after the first dose (Table 2). The amount of iodine needed to depress the thyroid accumulation of iodine by animals fed a Remington diet is not known. However, the maximal amount of iodine in any tracer dose (0.1 microgram as sodium iodide) was less than one tenth the minimal daily requirement of the thyroid of the normal rat (Chaikoff and Taurog, 1949; Dempsey and Astwood, 1943; Griesbach and Purves, 1945). Another possibility is that the thyroid was injured by radiation from the I^{131} in the tracer doses. Skanse (1948) in work on chicks found that a dose of 1.0 microcurie of I^{131} had no effect on the growth of the thyroid or its ability to collect iodine. A dose of 10.0 microcuries inhibited thyroid growth but had no effect on iodine collection.

In subsequent work with Merril and Evans, Skanse (1948) found that the growth of chick thyroids *collecting* 1.0 or 5.0 microcuries of I^{131} was significantly inhibited. The ability to concentrate iodine was not altered by 1.0 microcurie but was altered by 5.0 microcuries. If one can assume that the the rat's thyroid is no more sensitive to radiation than the chick's thyroid, it is not likely that the rats used in the present study received enough radiation to affect the uptake of iodine by the thyroid. It is possible that the decrease in uptake may represent a systematic error in the method, but if so, we have been unable to account for it.

Even though the nonhypophysectomized rats showed a decrease in the uptake of I^{131} after multiple tracer doses, the uptake was always appreciably greater than that of the hypophysectomized animals. The effect of repeated doses of radioactive iodine may have played some part in the decrease in uptake noted after hypophysectomy. Certainly, it was not the major factor, and decrease must be ascribed mainly to the absence of thyrotropic hormone. Part of the decrease may also be due to other factors as well, since hypophysectomy may affect renal and hemodynamic factors which determine to some extent the amount of iodine collected by the thyroid.

As a gross check on the accuracy of the data obtained by the *in vivo* method, the experiment was repeated using a different method of determining radioactivity. Groups of 16 to 20 rats each were hypophysectomized and sham-hypophysectomized. At one, three, five and seven days following operation, the animals and control groups were injected with 1 microcurie of I^{131}. Twenty-four hours after injection, the rats were killed, the thyroids were removed and the I^{131} in each gland was determined by a gamma ray counter. The mean thyroidal I^{131} of the control rats was 36 per cent of the dose. At one, three, five and seven days following operation, the sham-hypophysectomized rats collected 92, 103, 100 and 107 per cent respectively of the amount collected by the control rats, whereas the hypophysectomized rats collected 43, 40, 18 and 18 per cent of the amount collected by the controls. Thus, roughly the same results were obtained by a different method of determination in which the thyroidal I^{131} was measured directly and in which the effects of previous radiation were absent.

SUMMARY

The effect of hypophysectomy on the thyroidal collection of radiodioine was determined by an *in vivo* method following injection of 1.0 microcurie of I^{131}. Following hypophysectomy, the rat thyroid collects about 12 per cent as much iodine as before hypophysectomy. The defective collection of iodine occurs soon after operation and becomes maximal between five and six days after operation.

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THE *IN VIVO* DETERMINATION OF THE BIOLOGICAL DECAY OF THYROIDAL RADIOIODINE

A. ALBERT, M.D.

Endocrinology Laboratory, Section on Physiology, Mayo Clinic, Rochester, Minnesota

PRACTICALLY all experimental studies with radioiodine in the rat have been peformed by various methods which involve measurement of beta radiation of I^{131}. Although this method of determining radioiodine is accurate and sensitive, it involves killing the animal and preparing various tissues, chiefly the thyroid, in appropriate fashion for counting. Because of the variation among animals it is necessary to use fairly large numbers in any series of experiments. In vivo methods of measuring radioactive iodine depend on the detection of gamma radiation and are therefore less accurate and sensitive than those dependent on beta radiation. However, *in vivo* methods have the singular advantage that serial observations may be made on the same animal, which can be then subjected to a variety of conditions. This unusual advantage made it worth while to investigate the feasibility of an *in vivo* method for use in experimental studies on thyroidal function in the rat. It is the purpose of this paper to describe apparatus for such a method and to present one aspect of the studies in which it has been utilized; namely, the determination of the biological decay of thyroidal radioiodine.

MATERIAL AND METHODS

Male rats of either the Wistar or Sprague-Dawley strain varying from 1 month to 6 months of age were employed. They were maintained on a Remington diet and distilled water for one half to four months in order to increase the thyroidal collection of iodine. Before each experiment the hair on the ventral surface of the neck was completely removed by depilation, and a cross was made over the larynx with an indelible pencil. The thyroid is situated 1 to 2 mm. caudal to the larynx. From 0.5 to 10 microcuries I^{131} were injected subcutaneously in a volume of 1 cc. containing 0.1 microgram of sodium iodide. At varying intervals the rat was lightly anesthetized with ether and strapped to a board, which was then inserted under the counting table.

The counting table* (fig. 1) consists of a lead shield $\frac{3}{4}$ inch thick, 10 inches wide and 14 inches long. The shield is covered on the superior surface with a brass plate $\frac{1}{8}$ inch thick and is supported by aluminum legs $2\frac{1}{2}$ inches in length. Situated at 5 inches from

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one end of the shield and equally distant from the sides is a hole ¼ inch in diameter on the inferior surface and ½ inch in diameter on the superior surface. A removable stepped cylindrical plug made of the same materials as the shield fits snugly into the hole.

With the plug removed the board to which the animal is strapped is raised by means of a wooden wedge until the thyroid area, as indicated by the cross, is directly in the center of the hole. Sufficient pressure is exerted so that the skin is stretched lightly over the area of the hole, and thus the same geometric relationship to the tube in different

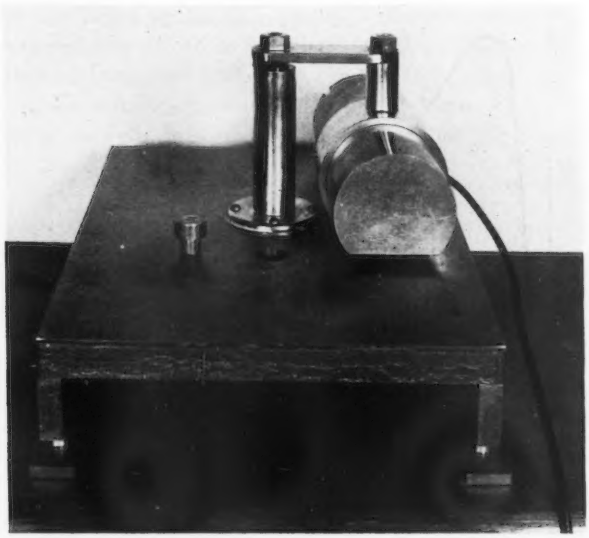

FIG. 1. Counting table, with stepped cylindrical plug removed and counting tube swung out of place.

observations is obtained as closely as possible. Continuous anesthesia is then maintained by the use of ether-soaked cotton.

The counting tube used was a disk electrode type, gamma ray tube (Sylvania Corporation GC 306) which was enclosed in a lead shield ½ inch thick (except over the hole in the table) and maintained on a pivot. The tube could then be swung into place over the hole into a constant position and the radiation over the thyroidal area determined. After a suitable count the tube was swung out of position, and the plug was inserted. Another count was obtained with the plug in place.

The first count represented a total radiation emanating not only from the thyroid but also from the extrathyroidal tissues, any radiation scattered or transmitted through the shield and the background of the tube itself. The second count represented the same sources of radiation with the exception that the count over the thyroidal area was essentially blocked out. The difference between the two counts, therefore, represents largely the radiation in the cylinder of tissue corresponding roughly to the hole in the shield. However, 95 per cent of this radiation emanates from the thyroid gland at least twenty-four hours or more after administration of radioactive iodine. This figure was determined

directly by measuring the radiation of rats into which radioiodine had been injected twenty-four hours previously and obtaining a second measurement immediately after the thyroids were surgically removed. In normal rats maintained on an iodine-deficient diet for two weeks, 18 to 33 counts per second (11 to 22 times the background count) are ordinarily obtained twenty-four hours after the injection of 1 microcurie of radioiodine.

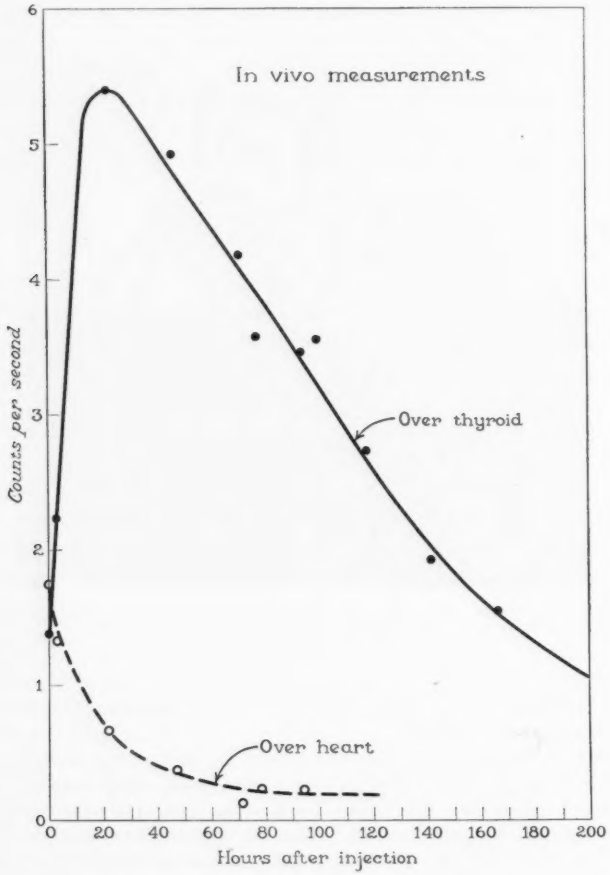

FIG. 2. *In vivo* measurements of I^{131} over the thyroidal area and over the heart at varying intervals after injection.

RESULTS

The results for 27 rats of the Wistar strain are reported here. One to 10 microcuries of radioactive iodide were injected subcutaneously, and counts were made twice daily for two weeks as described. An example of the data

obtained is shown in figure 2, where the counts per second over the thyroidal area corrected both for background and for physical decay of I^{131} are plotted versus time. Also shown is a similar count obtained over the heart. The usual type of curve for the accumulation of radioiodine in the thyroid is obtained, showing a rapid rise during the first day and then a slow decline over a period of ten days. Measurements were discontinued when the counts approached the background counting rate. The curve obtained over the heart probably represents disappearance of radioiodide from the blood and tissues of the body as a result of fixation in the thyroid and elimination in the urine and feces.

When the measurements at varying intervals were plotted on semilog paper (fig. 3), a straight line was obtained from which the proportional

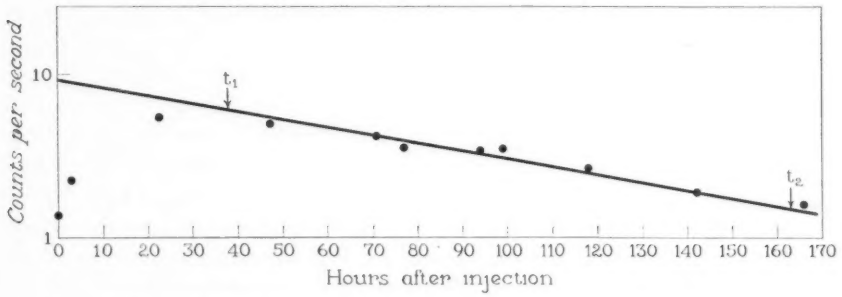

FIG. 3. Data of figure 2 plotted on semilog paper, showing method of calculation of proportional rate of diminution of I^{131} over thyroidal area.

rate of diminution of thyroidal I^{131} may be computed according to the formula: (Keating *et al.*, 1947).

$$\frac{\log Ct_1 - \log Ct_2}{0.434\ (t_2 - t_1)} \times 100 \times 24$$

The mean value $\pm S_{e.m.}$* for the 27 rats was 27.7 per cent ± 1.55 per cent per day.

DISCUSSION

The *in vivo* methods for determination of radioiodine suffer from certain disadvantages in that for many reasons the absolute proportions of the dose administered cannot be accurately and conveniently determined. However, certain of the technical errors committed in making any one determination are probably nullified when a series of similar determinations on the same animal are carried out. Thus, when an investigation entails

* Standard error of the mean.

establishment of the progression of events in an animal, which can then be experimentally altered, the *in vivo* method offers a method of study which is impossible by any other procedure.

The determination of the biological decay of thyroidal radioiodine is at best an approximation, for the reason that the total radiation measured includes a small proportion of extrathyroidal I^{131}. It is also in error to the extent that measurement at any time following the peak of accumulation may represent also a small amount of I^{131} that may have been secreted and reaccumulated by the thyroid. In spite of these difficulties, the disappearance of I^{131} over the thyroid as determined by the *in vivo* measurement is in good agreement with the rate of thyroidal secretion as calculated by various methods previously employed. In terms of thyroxine secreted per day from the thyroid of the rat, the previous estimates as determined by replacement therapy in thyroidectomized animals or by titration against the goitrogenic action of thiouracil range from 1 to 5 micrograms of d-l-thyroxine per day (Dempsey and Astwood, 1943; Reineke *et al.*, 1945). The amount of iodine in the thyroid of the rats reported in this study averages 6 micrograms. If all the thyroidal iodine is potentially capable of being converted into thyroxine, about 9 micrograms of thyroxine is potentially present in the thyroid. The proportional rate of diminution (27 per cent per day) would thus indicate a secretion rate of 2.5 micrograms of l-thyroxine, which is a good agreement with the value of 5 micrograms of d-l-thyroxine per day as determined by the thiouracil method.

SUMMARY

An *in vivo* apparatus for determining thyroidal I^{131} in the rat is described. The proportional rate of diminution of thyroidal I^{131} in male Wistar rats was determined by this method to be 27 per cent per day, which corresponds to a value of 2.5 micrograms of l-thyroxine per day. This value is in general agreement with previous methods for the determination of the secretion rate of the rat thyroid.

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NOTES AND COMMENTS

THE EFFECT OF HYPOPHYSECTOMY ON THE BIOLOGIC DECAY OF THYROIDAL RADIOIODINE

It has long been considered that following hypophysectomy the secretion of thyroid hormone ceases or at least is greatly diminished. The evidence for this belief is inferential, consisting chiefly of (1) the fall in plasma protein bound iodine, the decrease in basal metabolic rate and the atrophic appearance of the thyroid following hypophysectomy in animals and (2) the development of myxedema following pituitary failure in some patients. A method for measuring the effect of hypophysectomy on the rate of secretion of thyroid hormone was suggested by previous work (Albert, 1950), in which the biologic decay of thyroidal I^{131} was determined by an *in vivo* method. The biologic decay, or the rate of loss of I^{131} from the thyroidal area of the rat, is a general reflection of the endogenous secretion of thyroxine. The purpose of this communication is to establish the quantitative effect of hypophysectomy on the biologic decay of thyroidal I^{131}.

MATERIAL AND METHODS

Forty-five male rats of the Sprague-Dawley strain (40–50 gm.) were maintained on a Remington diet* and distilled water for two weeks. At the end of this time a tracer dose of 1 microcurie I^{131} was injected subcutaneously into each of the rats. Twenty-four hours later an *in vivo* measurement of the radiation over the thyroidal area was made according to the method previously described (Albert, 1950). The thyroidal I^{131} at this time, as is well known, is almost entirely in protein bound form (Chaikoff and Taurog, 1949); and thus thyroid hormone content of the thyroid is effectively labeled. Fifteen rats were maintained as controls, 15 were hypophysectomized and 15 were sham-hypophysectomized. The animals were returned to their cages and were maintained on the iodine deficient diet and 5 per cent glucose in distilled water. Measurements were made daily for sixteen days. The rates of diminution of thyroidal I^{131} were determined as previously described and expressed as a proportional rate of decline of thyroidal I^{131} in terms of per cent per day.

RESULTS

The data on individual rats are shown in Table 1. It is evident that the sham-hypophysectomized and the control rats had very similar rates of decline of the thyroidal I^{131}, the difference between them being statistically insignificant ($t=0.99$, $p=0.3$). On the other hand, the mean rate of diminution of thyroidal I^{131} in the hypophysectomized rats was 13 per cent of that of the controls ($t=10.1$, $p=<0.01$). Thus, a marked reduction in thyroidal secretion occurred as a result of hypophysectomy.

DISCUSSION

The results suggest that during the first sixteen days following hypophysectomy, thyroidal secretion, as reflected by the biologic decay of thyroidal I^{131}, does not cease

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* NaCl 50 gm., Brewers' yeast 10 gm., $CaCO_3$ 150 gm., corn 3800 gm., wheat gluten 1000 gm.

TABLE 1. PROPORTIONAL RATE OF DIMINUTION OF THYROIDAL I^{131} (PER CENT PER DAY)

Rat	Controls	Sham-hypophysectomized	Hypophysectomized
1	9.98	8.59	1.30
2	11.57	7.63	1.06
3	5.16	6.72	0.41
4	5.57	6.93	0.08
5	8.50	6.22	1.56
6	8.02	7.75	1.51
7	5.76	9.96	1.03
8	6.89	6.00	2.04
9	8.09	6.98	1.18
10	9.00	7.99	0.55
11	12.19	6.65	0.98
12	5.90		
Mean ±Sem*	8.05 ±0.67	7.31 ±0.37	1.06 ±0.17

* Standard error of the mean.

but rather falls to a level roughly one eighth that of normal animals maintained under the same environment. These results plus our unpublished observations on the collection of I^{131} showing that the thyroid of the hypophysectomized rat can collect one tenth to one sixth of the amount of I^{131} collected before hypophysectomy would indicate that both iodine collection and the discharge of hormone can occur in the absence of the pituitary but at a much reduced rate. Furthermore, the extent of the reduction of both thyroidal functions, collection and secretion, is affected by hypophysectomy to about the same extent. It is also of interest that the iodide trapping mechanism of the thyroid, as shown recently by Vanderlaan and Greer (1950), functions at about 10 per cent or so of its normal capacity a week or more after hypophysectomy.

SUMMARY

The biologic decay of thyroidal I^{131} is reduced to 13 per cent of normal during the first sixteen days following hypophysectomy.

RAYMOND V. RANDALL, NONA LORENZ AND A. ALBERT
Mayo Clinic, Rochester, Minnesota

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THE EFFECT OF ACUTE STARVATION ON THE THYROTROPHIC HORMONE LEVEL IN THE BLOOD OF THE RAT AND MOUSE*

It is well known that severe caloric restriction induces morphologic changes in various endocrine organs which can be correlated with peripheral effects indicative of altered hormone balance in the malnourished animal. The evidence suggests that the histophysiologic alterations occurring in the "target" glands during starvation states are referable primarily to differential effects on appropriate trophic mechanisms of the adenohypophysis. The regressive changes found in thyroid and gonads of starved animals simulate those found in hypophysectomy and can be reversed with pituitary substance (Mulinos and Pomerantz, 1941; Stephens, 1940; Stephens and Allen, 1941; D'Angelo *et al.*, 1942). Morphologic changes in the adrenal cortex, in contrast, suggest activation. Accordingly, it has been hypothesized that in caloric restriction there is a shift in the quality of trophic hormone activity in the adenohypophysis with augmented adrenocorticotrophic secretion probably occurring at the expense of less critical trophic horhomes (D'Angelo *et al.*, 1948; Boutwell *et al.*, 1948). Since starvation changes in target endocrine organs are thus ascribed to both excitatory and suppressive effects on the pituitary, substantiation of the hypothesis requires it to be demonstrated that blood trophic hormone levels are correlatively affected. The present experiments test one aspect of this concept by determining whether the involution of the thyroid gland in acute starvation can be accounted for by a change in the TSH concentration of the blood.

PROCEDURE

Adult male rats (290–460 gms.) and mice (25–35 gms.) were subjected to acute starvation for periods of 4–8 days by complete withdrawal of the normal laboratory ration. Water was not given during the starvation regime except in one group of four rats which received tap water "ad libitum." At approximately 30–35% body weight loss, the starved animals, together with normally fed controls, were lightly anesthetized, blood withdrawn by cardiac puncture, and the various endocrine organs quickly removed, weighed, and prepared for subsequent histologic examination. The bloods from animals in each group were pooled and the raw serum obtained was then bioassayed for thyrotrophic hormone with the stasis tadpole method (D'Angelo and Gordon, 1950).

RESULTS

The experimental results are summarized in table 1. Thyrotrophic hormone was detected in appreciable, and approximately equivalent, concentrations in the sera from both normally fed rats and mice of this study. The thyrotrophin content of sera (given in 6 injections; 0.05 cc. daily, alternate days) was sufficient to elevate thyroid cell heights in test tadpoles from 5.2 micra (water injected control value) to 6.9 and 7.0 micra for rat and mouse blood respectively. Activation of the thyroid with these sera was also accompanied by approximately equal hindlimb length increases. It was found that acute starvation led to a significant decrease in thyroid stimulating activity of blood. Thyroid cell heights with rat and mouse sera fell from the normal values previously indicated to 5.6 and 6.0 micra respectively, with corresponding decreases in hindlimb extension. (No

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appreciable differences in thyrotrophin levels of the blood were found in rats starved with or without water.) Microscopic examination of the thyroid glands in starved rats and mice revealed several major histological changes from the normal condition. There was an evident decrease in the height of the follicular epithelium in the majority of central follicles. Follicular distention occurred with the now hardened colloid showing minimal vacuolization. These histologic manifestations of reduced activity, however, did not approach the atrophic condition of hypophysectomy.

COMMENT

Evidence for suppression of the thyrotrophic mechanism in starvation has been largely indirect and inferential in nature. The concept has been made tenable by various experimental observations. Compensatory hypertrophy of the thyroid is greater in growing animals than in those losing weight (Loeb, 1919). The regressed gland can be restored to normal structure and function with physiologic doses of thyrotrophin (Stephens, 1940; D'Angelo *et al.*, 1950). The morphogenetic response of the thyroid to goitrogenic agents is decreased in partially starved rats (Gomez Mont, Paschkis, and Cantarow,

TABLE 1. THYROTROPHIC ACTIVITY OF SERA FROM NORMALLY FED AND ACUTELY STARVED MALE RATS AND MICE

Group	Mean initial body wgt.—gm. (Range)	Mean wgt. loss—% (Range)	Effect of sera on test tadpoles*	
			Mean hindlimb Increase (mm.)[1]	Thyroid cell Hgt. (micra)[2]
Rats—8 (Fed)	341 (285–424)		3.8 ± 0.2	6.9 ± 0.2
Rats—8 (Starved)	348 (290–460)	33.3 (29.4–37.0)	1.2 ± 0.1	5.6 ± 0.1
Mice—24 (Fed)	29.8 (25–33)		3.0 ± 0.3	7.0 ± 0.2
Mice—24 (Starved)	31.8 (26–38)	32.4 (24.2–40.1)	0.9 ± 0.3	6.0 ± 0.2

* Tadpoles in all groups received the same total volume of test sera (0.30 cc.).
[1] Mean hindlimb increase in mm. ± standard error of mean (initial hindlimb length similar in test groups).
[2] Thyroid cell height in micra ± standard error—based on measurements in 6–8 test animals.

1947). In underfed animals the reduced activity of the thyroid, as assessed by such criteria as weight response to thiouracil and by radioactive iodine uptake, appears to parallel the body weight loss (Meites and colleagues, 1949, 1950)—a result which suggests correspondingly reduced thyrotrophin activity.

It has already been shown that no endogenous thyrotrophin can be detected in the circulating blood of the hypophysectomized rat (D'Angelo and Gordon, 1950). The present demonstration of significant decreases in the titers of thyrotrophin in the blood of starved rats and mice presents direct evidence in support of the hypothesis that the involution of the thyroid in starvation is related to some alteration in the thyrotrophic mechanism of the adenohypophysis. The fact that blood thyrotrophin levels in starvation are diminished at a time when the thyroid is regressing is deemed quite significant, for if the inanition were operating primarily on the gland the resulting diminution in blood

thyroid hormone should elicit increased titers of circulating thyrotrophin. Although these results definitely implicate the hypophysis, it cannot be stated at present whether or not both production and release phases of thyrotrophic hormone secretion are affected in starvation.

SUMMARY AND CONCLUSION

The thyrotrophic hormone levels in the blood of adult male rats and mice are significantly reduced by acute starvation. The results are in conformity with the hypothesis that inanition depresses the thyrotrophic function of the pituitary.

SAVINO A. D'ANGELO

From the Department of Anatomy,
Jefferson Medical College,
Philadelphia,
Pennsylvania

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